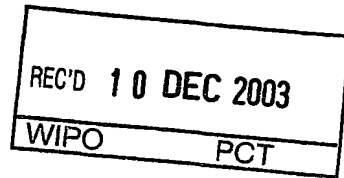




03 NOV 2003



INVESTOR IN PEOPLE



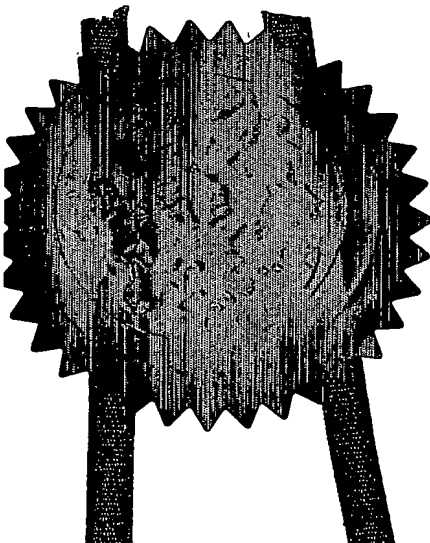
The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



R. Mahoney

Signed

Dated 16 September 2003

**PRIORITY
DOCUMENT**
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

Best Available Copy

Patents Form 1/77

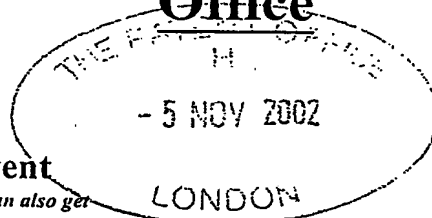
Patents Act 1977
(Rule 16)

The
Patent
Office

1/77

Request for grant of a patent

(See the notes on the back of this form. You can also get
an explanatory leaflet from the Patent Office to help
you fill in this form)



The Patent Office
Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference KLP/JN/PG5023

2. Patent application number
(The Patent Office will fill in his part)

0225788.9

05 NOV 2002

3. Full name, address and postcode of the or of
each applicant (underline all surnames)

Glaxo Group Limited
Glaxo Wellcome House, Berkeley Avenue,
Greenford, Middlesex UB6 0NN, Great Britain

Patents ADP number (*if you know it*)

If the applicant is a corporate body, give the
country/state of its incorporation

United Kingdom

473587003

4. Title of the invention

Vaccine

5. Name of your agent (*if you have one*)

Corporate Intellectual Property

"Address for service" in the United Kingdom
to which all correspondence should be sent
(*including the postcode*)

GlaxoSmithKline
Corporate Intellectual Property (CN9 25.1)
980 Great West Road
BRENTFORD
Middlesex TW8 9GS

Patents ADP number (*if you know it*)

7097960982003

6. If you are declaring priority from one or more
earlier patent applications, give the country
and the date of filing of the or each of
these earlier applications and (*if you know it*) the
or each application number

Country	Priority application number (<i>if you know it</i>)	Date of filing (<i>day / month / year</i>)
---------	--	---

7. If this application is divided or otherwise
derived from an earlier UK application,
give the number and the filing date of
the earlier application

Number of earlier application	Date of filing (<i>day / month / year</i>)
-------------------------------	---

8. Is a statement of inventorship and of right

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form.
Do not count copies of the same document

Continuation sheets of this form
Description
Claim(s)
Abstract
Drawings

41
4

22

only June

10. If you are also filing any of the following, state how many against each item.

Priority Documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*)

Request for substantive examination (*Patents Form 10/77*)

Any other documents
(please specify)

11.

We request the grant of a patent on the basis of this application

Signature K L Privett Date 5-Nov-02

12. Name and daytime telephone number of person to contact in the United Kingdom

K L Privett 020 80474451

Warning

After an application for a Patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission unless an application has been filed at least six weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

- If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505*
- Write your answers in capital letters using black ink or you may type them.*
- If there is not enough space for all relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be*

Vaccine

Field of the Invention

5 This invention relates to nucleic acid constructs, vectors comprising such constructs, methods of preparing the vectors and constructs and their use in prophylaxis or therapy, in particular therapeutic vaccines. The invention further relates to host cells comprising the constructs and vectors. The invention further relates to pharmaceutical formulations comprising the constructs and vectors and to the use of the constructs
10 and vectors in medicine. The invention relates in particular to DNA vaccines that are useful in the prophylaxis and treatment of HIV infections, more particularly when administered by particle mediated delivery.

Background to the Invention

15

HIV-1 is the primary cause of the acquired immune deficiency syndrome (AIDS) which is regarded as one of the world's major health problems. Although extensive research throughout the world has been conducted to produce a vaccine, such efforts thus far have not been successful.

20

The HIV envelope glycoprotein gp120 is the viral protein that is used for attachment to the host cell. This attachment is mediated by binding to two surface molecules of helper T cells and macrophages, known as CD4 and one of the two chemokine receptors CCR-4 or CXCR-5. The gp120 protein is first expressed as a larger
25 precursor molecule (gp160), which is then cleaved post-translationally to yield gp120 and gp41. The gp120 protein is retained on the surface of the virion by linkage to the gp41 molecule, which is inserted into the viral membrane.

30

The gp120 protein is the principal target of neutralizing antibodies, but unfortunately the most immunogenic regions of the proteins (V3 loop) are also the most variable parts of the protein. Therefore, the use of gp120 (or its precursor gp160) as a vaccine antigen to elicit neutralizing antibodies is thought to be of limited use for a broadly protective vaccine. The gp120 protein does also contain epitopes that are recognized

by cytotoxic T lymphocytes (CTL). These effector cells are able to eliminate virus-infected cells, and therefore constitute a second major antiviral immune mechanism. In contrast to the target regions of neutralizing antibodies some CTL epitopes appear to be relatively conserved among different HIV strains. For this reason gp120 and
 5 gp160 are considered to be useful antigenic components in vaccines that aim at eliciting cell-mediated immune responses (particularly CTL).

Non-envelope proteins of HIV-1 have been described and include for example internal structural proteins such as the products of the gag and pol genes and, other non-
 10 structural proteins such as Rev, Nef, Vif and Tat (Green et al., New England J. Med, 324, 5, 308 et seq (1991) and Bryant et al. (Ed. Pizzo), *Pediatr. Infect. Dis. J.*, 11, 5, 390 et seq (1992).

HIV Tat and Nef proteins are early proteins, that is they are expressed early in
 15 infection and in the absence of structural protein.

The Nef protein is known to cause the removal of CD4, the HIV receptor, from the cell surface, but the biological importance of this function is debated. Additionally Nef interacts with the signal pathway of T cells and induces an active state, which in
 20 turn may promote more efficient gene expression. Some HIV isolates have mutations in this region, which cause them not to encode functional protein and are severely compromised in their replication and pathogenesis in vivo.

The Tat gene gives rise to a number of differentially spliced transcripts at different
 25 times during infection. The first exon encodes an 86 amino acid protein which dominates early in infection. The second exon encodes an additional 14 amino acids, and this partially spliced form of tat is found late in infection. Both forms are fully functional transactivators, but the longer form also contains an RGD motif important for binding to $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. Tat binds to a short-stem loop structure, known
 30 as the transactivation response element (TAR), that is located at the 5' terminus of HIV RNAs, and up-regulates transcription from the HIV LTR at least 1000-fold. Tat has a role in promoting the elongation phase of HIV infection and stimulates the

These proteins have different roles e.g. the p2 protein has a proposed role in regulating activity of the protease and contributes to the correct timing of proteolytic processing.

5 The MA polypeptide is derived from the N-terminal, myristoylated end of p55. Most MA molecules remain attached to the inner surface of the virion lipid bilayer, stabilizing the particle. A subset of MA is recruited inside the deeper layers of the virion where it becomes part of the complex which escorts the viral DNA to the nucleus. These MA molecules facilitate the nuclear transport of the viral genome because a karyophilic signal on MA is recognized by the cellular nuclear import
10 machinery. This phenomenon allows HIV to infect non-dividing cells, an unusual property for a retrovirus.

The p24 (CA) protein forms the conical core of viral particles. Cyclophilin A has been demonstrated to interact with the p24 region of p55 leading to its incorporation
15 into HIV particles. The interaction between Gag and cyclophilin A is essential because the disruption of this interaction by cyclosporin A inhibits viral replication.

The NC region of Gag is responsible for specifically recognizing the so-called packaging signal of HIV. The packaging signal consists of four stem loop structures
20 located near the 5' end of the viral RNA, and is sufficient to mediate the incorporation of a heterologous RNA into HIV-1 virions. NC binds to the packaging signal through interactions mediated by two zinc-finger motifs. NC also facilitates reverse transcription.

25 The p6 polypeptide region mediates interactions between p55 Gag and the accessory protein Vpr, leading to the incorporation of Vpr into assembling virions. The p6 region also contains a so-called late domain which is required for the efficient release of budding virions from an infected cell.

30 The Pol gene encodes two proteins containing the two activities needed by the virus in early infection, the RT and the integrase protein needed for integration of viral DNA into cell DNA. The primary product of Pol is cleaved by the virion protease to yield the amino terminal RT peptide which contains activities necessary for DNA synthesis

production of full-length viral transcripts. Tat can affect the expression of a number of cellular genes and can activate the expression of a number of cellular genes including TNF, IL-2 and IL-6, and regulates expression of p53 and Bcl-2. Tat is produced in excess and is secreted from infected cells. This extra-cellular tat can enter other cells
 5 and may prime cells for infection by HIV or accelerate the rate of HIV replication in newly infected cells.

In a conference presentation (C. David Pauza, Immunization with Tat toxoid attenuates SHIV89.6PD infection in rhesus macaques, 12th Cent Gardes meeting,
 10 Marnes-La-Coquette, 26.10.1999), experiments were described in which rhesus macaques were immunised with Tat toxoid alone or in combination with an envelope glycoprotein gp160 vaccine combination (one dose recombinant vaccinia virus and one dose recombinant protein). The results observed showed that the presence of the envelope glycoprotein gave no advantage over experiments performed with Tat alone.

15

The Gag gene is translated from the full-length RNA to yield a precursor polyprotein which is subsequently cleaved into 3 – 5 capsid proteins; the matrix protein, capsid protein and nucleic acid binding protein and protease. (1. Fundamental Virology, Fields BN, Knipe DM and Howley M 1996 2. Fields Virology vol 2 1996).

20

The gag gene gives rise to the 55-kilodalton (kD) Gag precursor protein, also called p55, which is expressed from the unspliced viral mRNA. During translation, the N terminus of p55 is myristoylated, triggering its association with the cytoplasmic aspect of cell membranes. The membrane-associated Gag polyprotein recruits two copies of
 25 the viral genomic RNA along with other viral and cellular proteins that triggers the budding of the viral particle from the surface of an infected cell. After budding, p55 is cleaved by the virally encoded protease (a product of the pol gene) during the process of viral maturation into four smaller proteins designated MA (matrix [p17]), CA (capsid [p24]), NC (nucleocapsid [p9]), and p6.(4)

30

In addition to the 3 major Gag protein, all Gag precursors contain several other regions, which are cleaved out and remain in the virion as peptides of various sizes.

(RNA and DNA directed DNA polymerase, ribonuclease H) and carboxy terminal integrase protein. HIV RT is a heterodimer of full-length RT (p66) and a cleavage product (p51) lacking the carboxy terminal Rnase integrase domain.

5 RT is one of the most highly conserved proteins encoded by the retroviral genome. Two major activities of RT are the DNA Pol and Ribonuclease H. The DNA Pol activity of RT uses RNA and DNA as templates interchangeably and like all DNA polymerases known is unable to initiate DNA synthesis de novo, but requires a pre existing molecule to serve as a primer (RNA).

10

The Rnase H activity inherent in all RT proteins plays the essential role early in replication of removing the RNA genome as DNA synthesis proceeds. It selectively degrades the RNA from all RNA - DNA hybrid molecules. Structurally the polymerase and ribo H occupy separate, non-overlapping domains with the Pol

15

covering the amino two thirds of the Pol.

The p66 catalytic subunit is folded into 5 distinct subdomains. The amino terminal 23 of these have the portion with RT activity. Carboxy term to these is the Rnase H Domain.

20

After infection of the host cell, the retroviral RNA genome is copied into linear ds DNA by the reverse transcriptase that is present in the infecting particle. The integrase (reviewed in Skalka AM '99 Adv in Virus Res 52 271-273) recognises the ends of the viral DNA, trims them and accompanies the viral DNA to a host

25

chromosomal site to catalyse integration. Many sites in the host DNA can be targets for integration. Although the integrase is sufficient to catalyse integration in vitro, it is not the only protein associated with the viral DNA in vivo - the large protein - viral DNA complex isolated from the infected cells has been denoted the pre integration complex. This facilitates the acquisition of the host cell genes by progeny viral

30

genomes.

The integrase is made up of 3 distinct domains, the N terminal domain, the catalytic core and the c terminal domain. The catalytic core domain contains all of the requirements for the chemistry of polynucleotidyl transfer.

- 5 DNA vaccines usually consist of a bacterial plasmid vector into which is inserted a strong promoter, the gene of interest which encodes for an antigenic peptide and a polyadenylation/transcriptional termination sequence. The gene of interest may encode a full protein or simply an antigenic peptide sequence relating to the pathogen, tumour or other agent which it is intended to be protected against. The plasmid can be
- 10 grown in bacteria, such as for example *E.coli* and then isolated and prepared in an appropriate medium, depending upon the intended route of administration, before being administered to the host. Following administration the plasmid is taken up by cells of the host, or delivered directly into the host cells, where the encoded peptide is produced. The plasmid vector will preferably be made without an origin of replication
- 15 functional in eukaryotic cells, in order to prevent plasmid replication in the mammalian host and integration within chromosomal DNA of the animal concerned.

- There are a number of advantages of DNA vaccination relative to traditional vaccination techniques. First, it is predicted that because the proteins that are encoded
- 20 by the DNA sequence are synthesised in the host, the structure or conformation of the protein will be similar to the native protein associated with the disease state. It is also likely that DNA vaccination will offer protection against different strains of a virus, by generating a cytotoxic T lymphocyte response that recognises epitopes from conserved proteins. The technology also offers the possibility of combining diverse
- 25 immunogens into a single preparation to facilitate simultaneous immunisation in relation to a number of disease states.

- Helpful background information in relation to DNA vaccination is provided in Donnelly et al "DNA vaccines" Ann. Rev Immunol. 1997 15: 617-648, the disclosure
- 30 of which is included herein in its entirety by way of reference.

Doe et al (1994) Eur J Immunol, 24: 2369-2376 investigated how variations in glycosylation affected the CD8+ CTL response to gp120 and found that gp120

produced in mammalian CHO cells had a reduced ability to prime CTL responses when compared with insect or yeast cell-derived envelope proteins unless N-linked oligosaccharides were removed prior to immunization.

- 5 It has now been discovered that surprisingly, a DNA vector expressing gp120 without a secretion signal and which is thus not glycosylated or secreted from the cell is a more effective stimulator of CTL responses than a DNA vector expressing gp120 with its native secretion signal. Since the secretion signal is responsible for directing the gp120 to the intracellular site where glycosylation takes place, gp120 which lacks its
10 native secretion signal is not glycosylated. Moreover, with the presence of a non-structural HIV protein such as tat in a fusion protein with the non-glycosylated gp120, CTL responses to the gp120 are augmented. In contrast, tat in a fusion protein with normal gp120 prevents secretion but does not result in an augmented immune response.

15

Summary of the Invention

The present invention therefore provides novel constructs for use in nucleic acid vaccines for the prophylaxis and treatment of HIV infections and AIDS.

20

In one aspect the invention provides a polynucleotide which comprises a sequence encoding a gp120 molecule which is not glycosylated when expressed in a mammalian target cell, operably linked to a heterologous promoter.

- 25 Gp120 expressed in a mammalian cell will normally be glycosylated. According to the present invention the gp120 encoding sequence is adapted to prevent glycosylation in a mammalian target cell. Glycosylation may be prevented in a number of different ways, for example by removal of or mutation of the glycosylation sites or by removing the native secretion signal. Preferably in the polynucleotide construct according to the
30 invention the gp120 lacks a functional secretion signal.

The gp120 molecule is preferably expressed as a fusion protein, preferably comprising at least one HIV protein selected from a non-structural protein such as Nef, Tat, and reverse transcriptase (RT) and a structural protein such as Gag.

5 Particularly preferred is a fusion comprising gp120 and Tat. In such an embodiment the polynucleotide according to the invention comprises a gp120 encoding sequence linked to a Tat encoding sequence to encode a gp120 and Tat-containing fusion protein. Preferably the Tat sequence is mutated so that it encodes a biologically inactive Tat which lacks transactivation activity but which maintains its immunogenic
10 epitopes. Tat transactivation activity can be measured for example by an HIV LTR reporter system such as a CAT assay system in which the chloramphenicol-acetyl transferase reporter gene is under the control of the long terminal repeat of HIV-1. One specific CAT assay which is suitable uses the HL3T1 cell line which is described in Felber and Pavlakis (1988) Science 239: 184-187 and also used in Mischiati et al
15 (2001) Antisense Nucleic Acid Drug Dev Aug 11 (4): 209-17. HL3T1 is a HeLa cell line which contains stably integrated silent copies of HIV-1 LTR promoter linked to the CAT gene. These cells are transfected with DNA vectors containing the Tat gene, modified or not. CAT is produced upon presence of an active Tat and measured by ELISA.

20

One preferred mutated Tat sequence (originating from BH10 molecular clone) bears mutations in the active site region (Lys41→Ala) and in the RGD motif (Arg78→Lys and Asp80→Glu) (Virology 235: 48-64, 1997).

25 The fusion may contain further HIV sequences. It will be understood that for all of the HIV sequences included in the invention, these do not necessarily represent sequences encoding the full length or native proteins. Immunogenic derivatives such as truncated or otherwise altered e.g. mutated proteins are also contemplated, as are fragments which encode at least one HIV epitope, preferably a CTL epitope, typically
30 a peptide of at least 8 amino acids. Polynucleotides which encode a fragment of at least 8, for example 8-10 amino acids or up to 20, 50, 60, 70, 100, 150 or 200 amino acids in length are considered to fall within the scope of the invention as long as the

encoded oligo or polypeptide demonstrates HIV antigenicity. The HIV polypeptide molecules encoded by the polynucleotide sequences according to the invention preferably represent a fragment of at least 50% of the length of the native protein, which fragment may contain mutations but which retains at least one HIV epitope and demonstrates HIV antigenicity. The polynucleotide sequences are preferably codon optimised for mammalian cells, in line with preferred aspects of the invention.

In a particular embodiment the gp120 sequence is further linked to a Nef encoding sequence to encode a gp120, Tat and Nef-containing fusion protein, most preferably a gp120-Nef-Tat fusion. Optionally the Nef sequence is truncated to remove the sequence encoding the N terminal region i.e. removal of 30-85, preferably 60-85, preferably the N terminal 65 amino acids. Advantageously the Nef may be modified to remove one or more myristylation sites. For example the Gly 2 myristylation site may be removed by deletion or substitution. Alternatively or additionally the Nef may be modified to alter the dileucine motif of Leu 174 and Leu 175 by deletion or substitution of one or both leucines. The importance of the dileucine motif in CD4 downregulation is described e.g. in Bresnahan P.A. et al (1998) Current Biology, 8(22): 1235-8.

Additional HIV sequences may be included such as a Gag encoding sequence. Preferred Gag sequences comprise P17 and/or 24.

In another particular embodiment the fusion encoded by the polynucleotide according to the invention is a gp120-Gag-Nef-Tat fusion.

Preferably one or more of the HIV sequences included in the polynucleotide according to the invention encoding e.g. gp120, Nef, Tat or Gag is or are codon optimised.

Preferred polynucleotide sequences are selected from the group:

1. gp120 codon optimised, minus secretion signal
2. gp120 codon optimised, minus secretion signal – tr Nef
3. gp120 codon optimised, minus secretion signal – tr Nef – Tat

4. gp120 codon optimised, minus secretion signal – Nef - Tat
5. gp120 codon optimised, minus secretion signal – p17/24 Gag – tr Nef
6. gp120 codon optimised, minus secretion signal – p17/24 Gag – tr Nef - Tat
7. gp120 codon optimised, minus secretion signal - p17/24 gag - Nef-Tat
- 5 8. gp120 codon optimised, minus secretion signal - p17/24 gag - mNef-Tat
9. gp120 codon optimised, minus secretion signal - p17/24 gag - L1Nef-Tat
10. gp120 codon optimised, minus secretion signal - p17/24 gag - L2Nef-Tat
11. gp120 codon optimised, minus secretion signal - p17/24 gag - LLNef-Tat
12. gp120 codon optimised, minus secretion signal - p17/24 gag - mLLNef-Tat
- 10 13. gp120 codon optimised, minus secretion signal - p17/24 gag - mL1Nef-Tat
14. gp120 codon optimised, minus secretion signal - p17/24 gag - mL2Nef-Tat

mNef = deletion of G2 to give non-myristoylated Nef

L1 Nef = L174A mutation in Nef

- 15 L2Nef = L175A mutation in Nef

LLNef = L174A and L175A mutations in Nef

Preferably the promoter is the promoter from HCMV IE gene, more particularly wherein the 5' end of the promoter comprises exon 1 as described in WO 02/36792.

20

In another aspect the invention provides a vector comprising the polynucleotide sequences described herein. Preferably the vector is a double stranded DNA plasmid.

- 25 In yet another aspect the invention provides a polypeptide encoded by a polynucleotide or vector as described herein.

- 30 In a further aspect the invention provides pharmaceutical compositions comprising the nucleotide sequences and vectors and polypeptides described herein, together with a pharmaceutically acceptable excipient, diluent, carrier or adjuvant. In a preferred embodiment the polynucleotide, preferably in the form of a DNA vector and preferably comprising at least one codon optimised HIV sequence, is present in a composition comprising a plurality of particles, preferably beads such as gold beads, onto which the DNA is coated.

Delivery of polynucleotides according to the invention is preferably carried out by particle mediated delivery, particularly via a bombardment approach.

- 5 It is envisaged that the vectors according to the invention may be utilised with immunostimulatory agents, preferably but not necessarily administered at the same time as the vectors and preferably formulated together in the compositions according to the invention.
- 10 Immunostimulatory agents for use in the invention include, but this list is by no means exhaustive and does not preclude other agents: synthetic imidazoquinolines such as imiquimod [S-26308, R-837], (Harrison, et al. 'Reduction of recurrent HSV disease using imiquimod alone or combined with a glycoprotein vaccine', Vaccine 19: 1820-1826, (2001)); and resiquimod [S-28463, R-848] (Vasilakos, et al. 'Adjuvant activities
- 15 of immune response modifier R-848: Comparison with CpG ODN', Cellular immunology 204: 64-74 (2000).), Schiff bases of carbonyls and amines that are constitutively expressed on antigen presenting cell and T-cell surfaces, such as tucareol (Rhodes, J. et al. 'Therapeutic potentiation of the immune system by costimulatory Schiff-base-forming drugs', Nature 377: 71-75 (1995)), cytokine,
- 20 chemokine and co-stimulatory molecules as either protein or peptide, this would include pro-inflammatory cytokines such as GM-CSF, IL-1 alpha, IL-1 beta, TGF-alpha and TGF - beta, Th1 inducers such as interferon gamma, IL-2, IL-12, IL-15 and IL-18, Th2 inducers such as IL-4, IL-5, IL-6, IL-10 and IL-13 and other chemokine and co-stimulatory genes such as MCP-1, MIP-1 alpha, MIP-1 beta, RANTES, TCA-
- 25 3, CD80, CD86 and CD40L, , other immunostimulatory targeting ligands such as CTLA-4 and L-selectin, apoptosis stimulating proteins and peptides such as Fas, (49), synthetic lipid based adjuvants, such as vaxfectin, (Reyes et al., 'Vaxfectin enhances antigen specific antibody titres and maintains Th1 type immune responses to plasmid DNA immunization', Vaccine 19: 3778-3786) squalene, alpha- tocopherol,
- 30 polysorbate 80, DOPC and cholesterol, endotoxin, [LPS], Beutler, B., 'Endotoxin, 'Toll-like receptor 4, and the afferent limb of innate immunity', Current Opinion in Microbiology 3: 23-30 (2000)) ; CpG oligo- and di-nucleotides, Sato, Y. et al., 'Immunostimulatory DNA sequences necessary for effective intradermal gene

immunization', *Science* 273 (5273): 352-354 (1996). Hemmi, H. et al., 'A Toll-like receptor recognizes bacterial DNA', *Nature* 408: 740-745, (2000) and other potential ligands that trigger Toll receptors to produce Th1-inducing cytokines, such as synthetic Mycobacterial lipoproteins, Mycobacterial protein p19, peptidoglycan, teichoic acid and lipid A.

Certain preferred adjuvants for eliciting a predominantly Th1-type response include, for example, a Lipid A derivative such as monophosphoryl lipid A, or preferably 3-de-O-acylated monophosphoryl lipid A. MPL[®] adjuvants are available from Corixa Corporation (Seattle, WA; *see*, for example, US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555, WO 99/33488 and U.S. Patent Nos. 6,008,200 and 5,856,462. Immunostimulatory DNA sequences are also described, for example, by Sato et al., *Science* 273:352, 1996. Another preferred adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, MA); Escin; Digitonin; or *Gypsophila* or *Chenopodium quinoa* saponins.

According to a further aspect of the invention, a host cell comprising a polynucleotide sequence according to the invention, or an expression vector according to the invention, is provided. The host cell may be for example bacterial e.g. *E. coli*, mammalian e.g. human, or may be an insect cell. Mammalian cells comprising a vector according to the present invention may be cultured cells transfected *in vitro* or may be cells transfected *in vivo* by administration of the vector to the mammal.

By codon optimisation is meant that the DNA sequence is optimised to resemble the codon usage of genes in mammalian cells. In particular, the codon usage in the sequence is optimised to resemble that of highly expressed human genes.

The DNA code has 4 letters (A, T, C and G) and uses these to spell three letter "codons" which represent the amino acids the proteins encoded in an organism's genes. The linear sequence of codons along the DNA molecule is translated into the

linear sequence of amino acids in the protein(s) encoded by those genes. The code is highly degenerate, with 61 codons coding for the 20 natural amino acids and 3 codons representing "stop" signals. Thus, most amino acids are coded for by more than one codon - in fact several are coded for by four or more different codons.

5

Where more than one codon is available to code for a given amino acid, it has been observed that the codon usage patterns of organisms are highly non-random. Different species show a different bias in their codon selection and, furthermore, utilisation of codons may be markedly different in a single species between genes which are expressed at high and low levels. This bias is different in viruses, plants, bacteria and mammalian cells, and some species show a stronger bias away from a random codon selection than others. For example, humans and other mammals are less strongly biased than certain bacteria or viruses. For these reasons, there is a significant probability that a mammalian gene expressed in *E. coli* or a foreign or recombinant gene expressed in mammalian cells will have an inappropriate distribution of codons for efficient expression. It is believed that the presence in a heterologous DNA sequence of clusters of codons or an abundance of codons which are rarely observed in the host in which expression is to occur, is predictive of low heterologous expression levels in that host.

20

In an embodiment of the present invention there is provided a gp120 polynucleotide sequence which encodes a non-glycosylated gp120 amino acid sequence, wherein the codon usage pattern of the polynucleotide sequence resembles that of highly expressed mammalian genes. Preferably the polynucleotide sequence is a DNA sequence. Desirably the codon usage pattern of the polynucleotide sequence is typical of highly expressed human genes.

25

In the polynucleotides of the present invention, the codon usage pattern is altered from that typical of human immunodeficiency viruses to more closely represent the codon bias of the target organism, e.g. a mammal, especially a human. The "codon usage coefficient" is a measure of how closely the codon pattern of a given polynucleotide sequence resembles that of a target species. Codon frequencies can be derived from literature sources for the highly expressed genes of many species (see e.g. Nakamura

30

et.al. Nucleic Acids Research 1996, 24:214-215). The codon frequencies for each of the 61 codons (expressed as the number of occurrences occurrence per 1000 codons of the selected class of genes) are normalised for each of the twenty natural amino acids, so that the value for the most frequently used codon for each amino acid is set to 1 and the frequencies for the less common codons are scaled to lie between zero and 1. Thus each of the 61 codons is assigned a value of 1 or lower for the highly expressed genes of the target species. In order to calculate a codon usage coefficient for a specific polynucleotide, relative to the highly expressed genes of that species, the scaled value for each codon of the specific polynucleotide are noted and the geometric mean of all these values is taken (by dividing the sum of the natural logs of these values by the total number of codons and take the anti-log). The coefficient will have a value between zero and 1 and the higher the coefficient the more codons in the polynucleotide are frequently used codons. If a polynucleotide sequence has a codon usage coefficient of 1, all of the codons are "most frequent" codons for highly expressed genes of the target species.

According to the present invention, the codon usage pattern of the polynucleotide will preferably exclude codons representing < 10% of the codons used for a particular amino acid. A relative synonymous codon usage (RSCU) value is the observed number of codons divided by the number expected if all codons for that amino acid were used equally frequently. A polynucleotide of the present invention will preferably exclude codons with an RSCU value of less than 0.2 in highly expressed genes of the target organism. A polynucleotide of the present invention will generally have a codon usage coefficient for highly expressed human genes of greater than 0.3, preferably greater than 0.4, most preferably greater than 0.5. Codon usage tables for human can also be found in Genbank.

In comparison, a highly expressed beta actin gene has a RSCU of 0.747.

The codon usage table for a homo sapiens is set out below:

Codon usage for human (highly expressed) genes 1/24/91 (human_high.cod)

AmAcid	Codon	Number	/1000	Fraction	..
--------	-------	--------	-------	----------	----

5	Gly	GGG	905.00	18.76	0.24
	Gly	GGA	525.00	10.88	0.14
	Gly	GGT	441.00	9.14	0.12
	Gly	GGC	1867.00	38.70	0.50
10	Glu	GAG	2420.00	50.16	0.75
	Glu	GAA	792.00	16.42	0.25
	Asp	GAT	592.00	12.27	0.25
	Asp	GAC	1821.00	37.75	0.75
15	Val	GTG	1866.00	38.68	0.64
	Val	GTA	134.00	2.78	0.05
	Val	GTT	198.00	4.10	0.07
	Val	GTC	728.00	15.09	0.25
20	Ala	GCG	652.00	13.51	0.17
	Ala	GCA	488.00	10.12	0.13
	Ala	GCT	654.00	13.56	0.17
	Ala	GCC	2057.00	42.64	0.53
25	Arg	AGG	512.00	10.61	0.18
	Arg	AGA	298.00	6.18	0.10
	Ser	AGT	354.00	7.34	0.10
	Ser	AGC	1171.00	24.27	0.34
30	Lys	AAG	2117.00	43.88	0.82
	Lys	AAA	471.00	9.76	0.18
	Asn	AAT	314.00	6.51	0.22
	Asn	AAC	1120.00	23.22	0.78
35	Met	ATG	1077.00	22.32	1.00
	Ile	ATA	88.00	1.82	0.05
	Ile	ATT	315.00	6.53	0.18
	Ile	ATC	1369.00	28.38	0.77
40	Thr	ACG	405.00	8.40	0.15
	Thr	ACA	373.00	7.73	0.14
	Thr	ACT	358.00	7.42	0.14
	Thr	ACC	1502.00	31.13	0.57
45	Trp	TGG	652.00	13.51	1.00
	End	TGA	109.00	2.26	0.55
	Cys	TGT	325.00	6.74	0.32
	Cys	TGC	706.00	14.63	0.68

5	End	TAG	42.00	0.87	0.21
	End	TAA	46.00	0.95	0.23
	Tyr	TAT	360.00	7.46	0.26
	Tyr	TAC	1042.00	21.60	0.74
10	Leu	TTG	313.00	6.49	0.06
	Leu	TTA	76.00	1.58	0.02
	Phe	TTT	336.00	6.96	0.20
	Phe	TTC	1377.00	28.54	0.80
15	Ser	TCG	325.00	6.74	0.09
	Ser	TCA	165.00	3.42	0.05
	Ser	TCT	450.00	9.33	0.13
	Ser	TCC	958.00	19.86	0.28
20	Arg	CGG	611.00	12.67	0.21
	Arg	CGA	183.00	3.79	0.06
	Arg	CGT	210.00	4.35	0.07
	Arg	CGC	1086.00	22.51	0.37
25	Gln	CAG	2020.00	41.87	0.88
	Gln	CAA	283.00	5.87	0.12
	His	CAT	234.00	4.85	0.21
	His	CAC	870.00	18.03	0.79
30	Leu	CTG	2884.00	59.78	0.58
	Leu	CTA	166.00	3.44	0.03
	Leu	CTT	238.00	4.93	0.05
	Leu	CTC	1276.00	26.45	0.26
35	Pro	CCG	482.00	9.99	0.17
	Pro	CCA	456.00	9.45	0.16
	Pro	CCT	568.00	11.77	0.19
	Pro	CCC	1410.00	29.23	0.48

According to a further aspect of the invention, an expression vector is provided which comprises and is capable of directing the expression of a polynucleotide sequence according to the first aspect of the invention, in particular wherein the codon usage pattern of the gp120 polynucleotide sequence is typical of highly expressed mammalian genes, preferably highly expressed human genes. The vector may be suitable for driving expression of heterologous DNA in bacterial insect or mammalian

cells, particularly human cells. In one embodiment, the expression vector is p7313 (see Figure 1).

5 In a further aspect the invention provides a method of treating or preventing HIV infections, any symptoms or diseases associated therewith, comprising administering a safe and effective amount of a polynucleotide, a vector, a polypeptide or a pharmaceutical composition according to the invention.

10 Administration of the pharmaceutical composition may take the form of one or of more than one individual doses, for example as repeat doses of the same DNA plasmid, or in a "prime-boost" therapeutic vaccination regime. In one example of a prime-boost regime the "prime" vaccination may be via particle mediated DNA delivery of a polynucleotide according to the present invention, preferably incorporated into a plasmid vector, while the "boost" administration may be of a
15 recombinant viral vector comprising the same polynucleotide sequence, or the boosting may be carried out with the protein in adjuvant. Conversely the priming may be with the viral vector or with a protein formulation typically a protein formulated in adjuvant, and the boost a DNA vaccine of the present invention.

20 The invention further provides a process for the production of a polynucleotide as described herein comprising linking a nucleotide sequence encoding a non-glycosylated gp120 sequence and optionally a sequence encoding an HIV non-structural protein such as Tat, to a heterologous promoter sequence.

25 As discussed above, the present invention includes expression vectors that comprise the nucleotide sequences of the invention. Such expression vectors are routinely constructed in the art of molecular biology and may for example involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, such as for example polyadenylation signals which may be necessary, and which are
30 positioned in the correct orientation, in order to allow for protein expression. Other suitable vectors and how to construct them would be apparent to persons skilled in the art. By way of further example in this regard we refer to Sambrook *et al.* Molecular Cloning: a Laboratory Manual. 2nd Edition. CSH Laboratory Press. (1989).

Preferably, a polynucleotide of the invention, or for use in the invention in a vector, is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The
5 term “operably linked” refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence, such as a promoter, “operably linked” to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequence.

10

A nucleic acid sequence of the present invention may be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are discussed for example by Verme *et al*, Nature 1997, 389:239-242. Both viral and non-viral vector systems can be used. The vectors may be, for example, plasmids,
15 artificial chromosomes (e.g. BAC, PAC, YAC), virus or phage vectors provided with a origin of replication, optionally a promoter for the expression of the polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin or kanamycin resistance gene in the case of a bacterial plasmid or a resistance gene for a fungal vector. Vectors may
20 be used *in vitro*, for example for the production of DNA or RNA or used to transfect or transform a host cell, for example, a mammalian host cell e.g. for the production of protein encoded by the vector. The vectors may also be adapted to be used *in vivo*, for example in a method of DNA vaccination or of gene therapy.

25 Examples of suitable viral vectors include retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral, alpha-viral, Canarypox and vaccinia-viral based systems. Gene transfer techniques using these viruses are known to those skilled in the art. Retrovirus vectors for example may be used to stably integrate the polynucleotide of the invention into the host genome, although such recombination is not preferred.
30 Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression. Vectors capable of driving expression in insect cells (for example baculovirus vectors), in human cells, yeast or in bacteria may be employed in

order to produce quantities of the HIV protein encoded by the polynucleotides of the present invention, for example for use as subunit vaccines or in immunoassays.

Promoters and other expression regulation signals may be selected to be compatible
5 with the host cell for which expression is designed. For example, mammalian
promoters include the metallothionein promoter which can be induced in response to
heavy metals such as cadmium, and the β -actin promoter. Viral promoters such as the
SV40 large T antigen promoter, human cytomegalovirus (CMV) immediate early (IE)
promoter, rous sarcoma virus LTR promoter, adenovirus promoter, or a HPV
10 promoter, particularly the HPV upstream regulatory region (URR) may also be used.
All these promoters are well described and readily available in the art.

A preferred promoter element is the CMV immediate early promoter devoid of intron
A, but including exon 1. Accordingly there is provided a vector comprising a
15 polynucleotide of the invention under the control of HCMV IE early promoter. A
suitable HCMV IE promoter is described in WO 02/36792.

Non-viral based systems include direct administration of nucleic acids, microsphere
encapsulation technology poly(lactide-co-glycolide) and liposome-based systems.
20

The polynucleotides according to the invention have utility in the production by
expression of the encoded proteins, which expression may take place *in vitro*, *in vivo*
or *ex vivo*. The nucleotides may therefore be involved in recombinant protein
synthesis, for example to increase yields, or indeed may find use as therapeutic agents
25 in their own right, utilised in DNA vaccination techniques. Where the polynucleotides
of the present invention are used in the production of the encoded proteins *in vitro* or
ex vivo, cells, for example in cell culture, will be modified to include the
polynucleotide to be expressed. Such cells include transient, or preferably stable
mammalian cell lines. Particular examples of cells which may be modified by
30 insertion of vectors encoding for a polypeptide according to the invention include
mammalian HEK293T, CHO, HeLa, 293 and COS cells. Preferably the cell line
selected will be one which is stable. Expression may be achieved in transformed

oocytes. A polypeptide may be expressed from a polynucleotide of the present invention, in cells of a transgenic non-human animal, preferably a mouse. A transgenic non-human animal expressing a polypeptide from a polynucleotide of the invention is included within the scope of the invention.

5

The invention further provides a method of vaccinating a mammalian subject which comprises administering thereto an effective amount of a vaccine or vaccine composition according to the invention. Most preferably, expression vectors for use in DNA vaccines, vaccine compositions and immunotherapeutics will be plasmid
10 vectors.

DNA vaccines may be administered in the form of "naked DNA", for example in a liquid formulation administered using a syringe or high pressure jet, or DNA formulated with liposomes or an irritant transfection enhancer, or by particle mediated
15 DNA delivery (PMDD or particle mediated immunotherapeutic delivery PMID) as described in more detail herein. All of these delivery systems are well known in the art. The vector may be introduced to a mammal for example by means of a viral vector delivery system.

20 The compositions of the present invention can be delivered by a number of routes such as intramuscularly, subcutaneously, intraperitoneally, intravenously or mucosally.

The invention further provides an intradermal delivery device comprising a pharmaceutical composition described herein.

25

In a preferred embodiment, the composition is delivered intradermally. In particular, the composition is delivered by means of a gene gun particularly using particle bombardment administration techniques which involve coating the vector on to beads (eg gold beads) which are then administered under high pressure into the epidermis.

30 This is described, for example, in Haynes et al, J Biotechnology 44: 37-42 (1996).

Numerous methods of carrying out a particle bombardment approach are known, see for example WO 91/07487. In one illustrative example, gas-driven particle

acceleration can be achieved with devices such as those manufactured by Powderject Pharmaceuticals PLC (Oxford, UK) and Powderject Vaccines Inc. (Madison, WI), some examples of which are described in U.S. Patent Nos. 5,846,796; 6,010,478; 5,865,796; 5,584,807; and EP Patent No. 0500 799. This approach offers a needle-free delivery approach wherein a dry powder formulation of microscopic particles, such as polynucleotide, are accelerated to high speed within a helium gas jet generated by a hand held device, propelling the particles into a target tissue of interest, typically the skin. The particles are preferably gold beads of a 0.4 – 4.0 μm , more preferably 0.6 – 2.0 μm diameter and the DNA conjugate coated onto these and then encased in a cartridge or cassette for placing into the delivery device.

In a related embodiment, other devices and methods that may be useful for gas-driven needle-less injection of compositions of the present invention include those provided by Bioject, Inc. (Portland, OR), some examples of which are described in U.S. Patent Nos. 4,790,824; 5,064,413; 5,312,335; 5,383,851; 5,399,163; 5,520,639 and 5,993,412.

The vectors which comprise the nucleotide sequences encoding antigenic peptides are administered in such amount as will be prophylactically or therapeutically effective. The quantity to be administered, is generally in the range of one picogram to 1 milligram, preferably 1 picogram to 10 micrograms for particle-mediated delivery, and 100 nanograms to 10 milligrams for other routes of nucleotide per dose. The exact quantity may vary considerably depending on the weight of the patient being immunised and the route of administration.

It is possible for the immunogen component comprising the nucleotide sequence encoding the antigenic peptide, to be administered on a one off basis or to be administered repeatedly, for example, between 1 and 7 times, preferably between 1 and 4 times, at intervals between about 1 day and about 18 months. However, this treatment regime will be significantly varied depending upon the size of the patient concerned, the amount of nucleotide sequence administered, the route of administration, and other factors which would be apparent to a skilled medical

practitioner. The patient may receive one or more other anti HIV retroviral drugs as part of their overall treatment regime. Additionally the nucleic acid immunogen may be administered with an adjuvant.

- 5 The adjuvant component specified herein can similarly be administered via a variety of different administration routes, such as for example, via the oral, nasal, pulmonary, intramuscular, subcutaneous, intradermal or topical routes. Preferably, the adjuvant component is administered via the intradermal or topical route, most preferably by a topical route. This administration may take place between about 14 days prior to and
10 about 14 days post administration of the nucleotide sequence, preferably between about 1 day prior to and about 3 days post administration of the nucleotide sequence.

- The adjuvant component is, in one embodiment, administered substantially simultaneously with the administration of the nucleotide sequence. By “substantially
15 simultaneous” what is meant is that administration of the adjuvant component is preferably at the same time as administration of the nucleotide sequence, or if not, it is at least within a few hours either side of nucleotide sequence administration. In the most preferred treatment protocol, the adjuvant component will be administered substantially simultaneously with administration of the nucleotide sequence.
- 20 Obviously, this protocol can be varied as necessary, in accordance with the type of variables referred to above. It is preferred that the adjuvant is a 1H – imidazo [4,5c] quinoline – 4 – amine derivative such as imiquimod. Typically imiquimod will be presented as a topical cream formulation and will be administered according to the above protocol.

25

- Once again, depending upon such variables, the dose of administration of the derivative will also vary, but may, for example, range between about 0.1 mg per kg to about 100 mg per kg, where “per kg” refers to the body weight of the mammal concerned. This administration of the 1H-imidazo[4,5-c]quinolin-4-amine derivative
30 would preferably be repeated with each subsequent or booster administration of the nucleotide sequence. Most preferably, the administration dose will be between about 1 mg per kg to about 50 mg per kg. In the case of a “prime-boost” scheme as described herein, the imiquimod or other 1H-imidazo[4,5-c]quinolin-4-amine

derivative may be administered with either the prime or the boost or with both the prime and the boost.

While it is possible for the adjuvant component to comprise only 1H-imidazo[4,5-c]quinolin-4-amine derivatives to be administered in the raw chemical state, it is preferable for administration to be in the form of a pharmaceutical formulation. That is, the adjuvant component will preferably comprise the 1H-imidazo[4,5-c]quinolin-4-amine combined with one or more pharmaceutically acceptable carriers, and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with other ingredients within the formulation, and not deleterious to the recipient thereof. The nature of the formulations will naturally vary according to the intended administration route, and may be prepared by methods well known in the pharmaceutical art. All methods include the step of bringing into association a 1H-imidazo[4,5-c]quinolin-4-amine derivative with an appropriate carrier or carriers. In general, the formulations are prepared by uniformly and intimately bringing into association the derivative with liquid carriers or finely divided solid carriers, or both, and then, if necessary, shaping the product into the desired formulation. Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a pre-determined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, lubricating, surface active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient.

Formulations for injection via, for example, the intramuscular, intraperitoneal, intradermal, or subcutaneous administration routes include aqueous and non-aqueous sterile injection solutions which may contain antioxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described. Formulations suitable for pulmonary administration via the buccal or nasal cavity are presented such that particles containing the active ingredient, desirably having a diameter in the range of 0.5 to 7 microns, are delivered into the bronchial tree of the recipient. Possibilities for such formulations are that they are in the form of finely comminuted powders which may conveniently be presented either in a piercable capsule, suitably of, for example, gelatine, for use in an inhalation device, or alternatively, as a self-propelling formulation comprising active ingredient, a suitable liquid propellant and optionally, other ingredients such as surfactant and/or a solid diluent. Self-propelling formulations may also be employed wherein the active ingredient is dispensed in the form of droplets of a solution or suspension. Such self-propelling formulations are analogous to those known in the art and may be prepared by established procedures. They are suitably provided with either a manually-operable or automatically functioning valve having the desired spray characteristics; advantageously the valve is of a metered type delivering a fixed volume, for example, 50 to 100 μL , upon each operation thereof.

In a further possibility, the adjuvant component may be in the form of a solution for use in an atomiser or nebuliser whereby an accelerated airstream or ultrasonic agitation is employed to produce a fine droplet mist for inhalation.

Formulations suitable for intranasal administration generally include presentations similar to those described above for pulmonary administration, although it is preferred for such formulations to have a particle diameter in the range of about 10 to about 200 microns, to enable retention within the nasal cavity. This may be achieved by, as
5 appropriate, use of a powder of a suitable particle size, or choice of an appropriate valve. Other suitable formulations include coarse powders having a particle diameter in the range of about 20 to about 500 microns, for administration by rapid inhalation through the nasal passage from a container held close up to the nose, and nasal drops comprising about 0.2 to 5% w/w of the active ingredient in aqueous or oily solutions.

10 In one embodiment of the invention, it is possible for the vector which comprises the nucleotide sequence encoding the antigenic peptide to be administered within the same formulation as the 1H-imidazo[4,5-c]quinolin-4-amine derivative. Hence in this embodiment, the immunogenic and the adjuvant component are found within the same formulation.

15

In one embodiment the adjuvant component is prepared in a form suitable for biolistic administration, and is administered via that route substantially simultaneously with administration of the nucleotide sequence. For preparation of formulations suitable for use in this manner, it may be necessary for the 1H-imidazo[4,5-c]quinolin-4-amine
20 derivative to be lyophilised and adhered onto, for example, particles such as gold beads which are suited for biolistic administration.

In an alternative embodiment, the adjuvant component may be administered as a dry powder, via high pressure gas propulsion.

25

Even if not formulated together, it may be appropriate for the adjuvant component to be administered at or about the same administration site as the nucleotide sequence.

Other details of pharmaceutical preparations can be found in Remington's
30 Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania (1985), the disclosure of which is included herein in its entirety, by way of reference.

Suitable techniques for introducing the naked polynucleotide or vector into a patient

also include topical application with an appropriate vehicle. The nucleic acid may be administered topically to the skin, or to mucosal surfaces for example by intranasal, oral, intravaginal or intrarectal administration. The naked polynucleotide or vector may be present together with a pharmaceutically acceptable excipient, such as
5 phosphate buffered saline (PBS). DNA uptake may be further facilitated by use of facilitating agents such as bupivacaine, either separately or included in the DNA formulation. Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding which is described in US 5,697,901.

10 Uptake of nucleic acid constructs may be enhanced by several known transfection techniques, for example those including the use of transfection agents. Examples of these agents include cationic agents, for example calcium phosphate and DEAE-Dextran and lipofectants, for example lipofectam and transfectam. The dosage of the
15 nucleic acid to be administered can be altered.

A nucleic acid sequence of the present invention may also be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are discussed for example by Verme *et al*, Nature 1997, 389:239-242. Both viral and
20 non-viral vector systems can be used and are described above. Viral and non-viral delivery systems may be combined where it is desirable to provide booster injections after an initial vaccination, for example an initial "prime" DNA vaccination using a non-viral vector such as a plasmid followed by one or more "boost" vaccinations using a viral vector or non-viral based system. Similarly the invention contemplates
25 prime boost systems with the polynucleotide of the invention, followed by boosting with protein in adjuvant or vice versa.

A nucleic acid sequence of the present invention may also be administered by means of transformed cells. Such cells include cells harvested from a subject. The naked
30 polynucleotide or vector of the present invention can be introduced into such cells *in vitro* and the transformed cells can later be returned to the subject. The polynucleotide of the invention may integrate into nucleic acid already present in a cell by homologous recombination events. A transformed cell may, if desired, be

grown up *in vitro* and one or more of the resultant cells may be used in the present invention. Cells can be provided at an appropriate site in a patient by known surgical or microsurgical techniques (e.g. grafting, micro-injection, etc.)

- 5 The pharmaceutical compositions of the present invention may include adjuvant compounds as detailed above, or other substances which may serve to increase the immune response induced by the protein which is encoded by the DNA. These may be encoded by the DNA, either separately from or as a fusion with the antigen, or may be included as non-DNA elements of the formulation. Examples of adjuvant-type
- 10 substances which may be included in the formulations of the present invention include ubiquitin, lysosomal associated membrane protein (LAMP), hepatitis B virus core antigen, FLT3-ligand (a cytokine important in the generation of professional antigen presenting cells, particularly dendritic cells) and other cytokines such as IFN- γ and GMCSF. Other preferred adjuvants include imiquimod and resiquimod and
- 15 tucarasol, imiquimod being particularly preferred.

In a particular embodiment of the invention there is provided the use of a nucleic acid molecule as herein described for the treatment or prophylaxis of HIV infection, administered with imiquimod. The imiquimod is preferably administered topically,

20 whereas the nucleic acid molecule is preferably administered by means of particle mediated delivery.

Accordingly the present invention also provides a method of treating a subject suffering from or susceptible to HIV infection, comprising administering a nucleic acid molecule as herein described and imiquimod.

25

The present invention will now be described by reference to the following examples:

EXAMPLES

Example 1: Plasmid Construction

5 1.1 Construction of gp120 containing plasmid

Recombinant gp120 glycoprotein described in the following examples is a synthetic form of the gp120 envelope protein of HIV-1 isolate W61D.

10 Codon Optimised (pgp120c):

The gene sequence was based on the gp120 sequence from the HIV-1 isolate W61D. This has a Codon Usage Coefficient of 0.297. Optimisation was performed using SynGene 2d, resulting in a CUC of 0.749. The sequence was split into 40 overlapping
15 oligonucleotides, PCR assembled and recovered using the end primers. The gene was cloned into vector p7313-ie (shown in Figure 1) as a NotI-BamHI fragment and sequenced. Restriction fragments from three initial clones were combined to generate a single correct clone. The amino acid sequence and codon optimised DNA sequence are given in Figure 2.

20

1.2 Generation of Nef/Tat containing plasmids

Nef/Tat (pNTm and ptrNTm)

The gene for the Nef/Tat fusion protein was provided in plasmid pRIT15244 (Figure
25 3). The plasmid pRIT 15244 is identical to pRIT 14913 described below except that the His tail has been deleted.

General

30 The Nef gene from the Bru/Lai isolate (Cell 40: 9-17, 1985) was selected for the constructs since this gene is among those that are most closely related to the consensus Nef.

The starting material for the Bru/Lai Nef gene was a 1170bp DNA fragment cloned on the mammalian expression vector pcDNA3 (pcDNA3/Nef).

- 5 The Tat gene originates from the BH10 molecular clone. This gene was received as an HTLV III cDNA clone named pCV1 and described in Science, 229, p69-73, 1985. This *tat* gene bears mutations in the active site region (Lys41→Ala) and in RGD motif (Arg78→Lys and Asp80→Glu) (Virology 235: 48-64, 1997).
- 10 The mutant *tat* gene was received as a cDNA fragment subcloned between the EcoRI and HindIII sites within a CMV expression plasmid (pCMVLys41/KGE)

Construction of vector pRIT14597 (encoding Nef-His protein).

15

The *nef* gene was amplified by PCR from the pcDNA3/Nef plasmid with primers 01 and 02.

NcoI

20 PRIMER 01: 5'ATCGTCCATG.GGT.GGC.AAG.TGG.T 3'

SpeI

PRIMER 02: 5' CGGCTACTAGTGCAGTTCTTGAA 3'

- 25 The integrative vector PHIL-D2 (INVITROGEN) was used. This vector was modified in such a way that expression of heterologous protein starts immediately after the native ATG codon of the AOX1 gene and will produce recombinant protein with a tail of one glycine and six histidines residues. This PHIL-D2-MOD vector was constructed by cloning an oligonucleotide linker between the adjacent AsuII and
- 30 EcoRI sites of PHIL-D2 vector. In addition to the His tail, this linker carries NcoI, SpeI and XbaI restriction sites between which *nef*, *tat* and *nef-tat* fusion were inserted.

The *nef* PCR fragment obtained and the integrative PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14597.

5

Construction of vector pRIT14913 (encoding fusion Nef-Tat mutant-His).

To construct pRIT14913, the *tat* mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with primers 03 and 04.

10

SpeI

PRIMER 03: 5' ATCGTACTAGT.GAG.CCA.GTA.GAT.C 3'

SpeI

15 PRIMER 04: 5' CGGCTACTAGTTTTCCTTCGGGCCT 3'

20

The PCR fragment obtained and the plasmid pRIT14597 (expressing Nef-His protein) were both digested by SpeI restriction enzyme, purified on agarose gel and ligated to create the integrative plasmid pRIT14913.

1.3 Generation of PMID vectors for gp120 and Nef/Tat:

25

gp120: Codon-optimised gp120 was provided as described above.

Nef/Tat (pNTm and ptrNTm):

30

The gene for the Nef/Tat fusion protein was provided in plasmid pRIT15244 described above. The Tat in this plasmid contains three mutations to inactivate the transactivation function. The fusion contains full length Nef which has an immune modulatory function (Collins and Baltimore (1999)) that may be abrogated by N-terminal truncation. Therefore constructs were generated for both full length

Nef/mutant Tat(pNTm) and truncated Nef/mutant Tat(ptrNTm), in which the first 65 amino acids of Nef were removed. These sequences were PCR amplified from pRIT15244 using primers:

5 5'Nef GAATTCGCGGCCGCCATGGGTGGCAAGTGGTCAAAAAG
 5'trNef GAATTCGCGGCCGCCATGGTGGGTTTTCCAGTCACACC
 3'Tat GAATTCGGATCCTTATTCCTTCGGGCCTGTCGGG

The genes were cloned into vector p7313-ie as NotI-BamHI fragments and sequenced.

10 PNTm and ptrNTm and the Nef/Tat and truncated Nef/Tat sequences are shown in Figures 4 and 5.

Dual expression vectors: (pRIX1 and pRIX2)

15 The Nef/Tat and trNef/Tat expression cassettes were excised as ClaI-XmnI restriction fragments, and ligated into the ClaI and blunted Sse8387 I sites of the vector containing the codon optimised gp120 (pgp120c) to provide single plasmids for expression of both proteins (pRIX1 and pRIX2 respectively).

20 Composition of plasmid p7313-ie (Figure 1)

The plasmid was constructed by replacing the beta-lactamase gene containing Eam1105I - PstI fragment of pUC19 (available from Amersham Pharmacia Biotech UK Ltd., Amersham Place, Little Chalfont, Bucks, HP7 9NA) with an EcoRI
 25 fragment of pUC4K (Amersham-Pharmacia) containing the Kanamycin resistance gene, following blunt ending of both fragments using T4 DNA polymerase. The human Cytomegalovirus IE1 promoter /enhancer, Intron A, was derived from plasmid JW4303 obtained from Dr Harriet Robinson, University of Massachusetts, and inserted into the SalI site of pUC19 as a XhoI -SalI fragment, incorporating the
 30 bovine growth hormone polyadenylation signal. Deletion of the 5' SalI-BanI fragment from the promoter generated the minimal promoter used in the vector (WO00/23592 - Powderject Vaccines Inc.). HBV Surface antigen 3'UTR was derived from Hepatitis B Virus, serotype adw, in the vector pAM6 (Moriarty et al., Proc.Natl.Acad.Sci. USA,

78, 2606-10, 1981). pAM6 (pBR322 based vector) was obtained from the American Type Culture Collection, catalogue number ATCC 45020. The 3'UTR was inserted 5' to the polyadenylation signal as a 1.4kb BamHI fragment, blunt ended for insertion to remove the BamHI sites. In a series of steps (including digestion with *Bgl* II, Klenow polymerase treatment, digestion with *Bst*X I, digestion with *Nco* I, treatment with mung bean nuclease to remove overhang and further digestion with *Bst*X I), modifications were made to the region between the 3'untranslated enhancer region of the HBV S gene and bGHpA signal to remove all open reading frames of greater than 5 codons between the X gene promoter and the bGHpA signal. This resulted in deletion of sequence encoding the translatable portion of the X protein (9 amino acids) and the X gene start codon. The bovine growth hormone polyadenylation signal was substituted with the rabbit beta globin polyadenylation signal. The 5'non-coding and coding sequences of the S antigen were excised and replaced with an oligonucleotide linker to provide multiple cloning sites as shown to produce plasmid p7313-PL.

Hind---NotI-- EcoRV- -NdeI- -BamHI
 AGCTTGCGGCCGCTAGCGATATCGGTACCATATGTCGACGGATCC....
ACGCCGGCGATCGCTATAGCCATGGTCTACAGCTGCCTAGGCCGG
 -NheI- -KpnI- -Sall- ΔNotI

This polylinker was further extended by insertion of an additional oligonucleotide linker between the KpnI and Sall sites:

AspI- -MunI- NaeI- NdeI-- BglII-
 GTACCGGTCAATTGGCGCCGGCGCGCCATATGACGTCAGATCTG----
 ----GCCAGTTAACCGCGGCCGCGCGGTATACTGCAGTCTAGACAGCT
 --AgeI- -NarI-- AatII- Sall

The ColE1 cer sequence was obtained from a subclone from plasmid pDAH212 from David Hodgeson (Warwick University) and amplified by PCR using primers to place EcoRI restriction sites at the ends of the sequence. The cer sequence was then inserted

into the EcoRI site of p7313-PL to produce plasmid p7313-PLc. The sequence of the amplified cer was verified against the Genbank entry M11411.

The HBV 3'UTR sequence between the promoter and polyadenylation signal was removed by PCR amplification of the polyadenylation signal using primers:

5 sense: CCATGGATCCGATCTTTTCCCTCTGCC

antisense: GTTAGGGTGAAAAGCTTCCGAGTGAGAGACAC

The resulting product was cut with BamHI and XmnI and used to replace the corresponding fragment containing both the polyadenylation signal and the 3'UTR.

10 The Intron A sequence was removed from the plasmid by PCR amplification of the CMV promoter/enhancer using primers:

sense: GCTAGCCTGCAGGCTGACCGCCCAACGAC

antisense: GTTCTCCATCGCGGCCGCACTCTTGGCACGGGG

The resulting product was cut with Sse8387 I and NotI, and inserted back into the Sse8387 I and NotI sites of the parental vector.

15

Example 2: Modification of gp120 and Nef/Tat(mut) expression vectors

gp120 constructs were modified to reduce secretion of the protein.

20

Generation of constructs:

gp120 without a secretion signal (dsgp120, pRix 12 – see Figure 2 and 6)

The gp120 gene was PCR amplified from pgp120c using the following primers:

25

5'120ds: 5'GAATTCGCGGCCGCCATGGCCGAGCAGCTGTGGGTCACC

L01:

5'GAATTCGGATCCTCATCTCTGCACGACGCGGCGCTTGGCCCGGGT
GGGGGCCACG

30

Fragments were amplified using PWO DNA polymerase (Roche) and the cycle:

95°C (30s) 95°C(30s) 50°C(30s) 72°C(90s) 72°C(120s) 4°C(hold)

\ _____ repeat x20 _____ /

- 5 The products were cut with NotI and BamHI and cloned into p7313-ie to give pRix12 (Figure 6).

Results

- 10 In 293T cells the vector pRIX12, which lacks the secretion signal, makes a good amount of a 60kDa non-glycosylated protein that is not secreted.

Example 3: Construction of vectors for expression of gp120 and Nef/Tat(mut) from a single plasmid

Vector construction:

The gp120 Nef/Tat(m) constructs were generated by PCR stitching the gp120 and Nef/Tat(m) or trNef/Tat(m) orfs.

5' and 3' Gp120, 5' and 3' Nef/Tat(m) and 5'trNef/Tat were amplified from pRix1. 3'trNef/Tat(m) was amplified from pNTm. The following primers were used:

3'120: (antisense to): GCCAAGCGCCGCGTCGTGCAGAGA

25 5'120/NT: GCCAAGCGCCGCGTCGTGCAGAGAATGGGTGGCAAGTGGTCAAAAAGT

3'NT (antisense to): GGGGAGCCGACAGGCCCGAAGGAA

5'NT/120:

GGGGAGCCGACAGGCCCGAAGGAAATGAAGGTCAAGGAGACCAGAAAG

5'120/trN:

30 GCCAAGCGCCGCGTCGTGCAGAGAATGGTGGGTTTTCCAGTCAC

5'trNef:

GAATTCGCGGCCGCCATGGTGGGTTTTCCAGTCACACC

L01:

GAATTCGGATCCTCATCTCTGCACGACGCGGCGCTTGGCCCCGGGTG
GGGGCCACG

L02:

5 ACCACCTTGTACTTGTACAGCTCGCTCCGCCAGTTATCCCTCATGTC
GCCGCCGCCGGGC

Fragments were amplified using PWO DNA polymerase (Roche) and the cycle:

10 95°C (60s) 95°C(30s) 55°C(30s) 72°C(120s) 72°C(120s) 4°C(hold)
 \ ____ repeat x20 ____ /

Primer L1 was used as the 3' primer for 3'gp120. However there were problems using
this primer when stitching Nef/Tat or trNef/T to the 5' end of gp120 so primer L2 was
15 used instead.

The stitched gp120-N/Tm and gp120-trN/Tm fragments were cut with NotI and
BamHI and cloned into similarly cut p7313-*ie*. Due to the use of primer L2 rather than
L1 the N/Tm-gp120 and trN/Tm-gp120 fragments lacked a BamHI site, so these were
20 cut with NotI and AccI, and cloned into similarly cut pgp120c. All inserts were fully
verified by sequencing. The plasmids were designated pRix6 (gp120c NefTat^m) and
pRix11 (gp120c trNefTat^m).

25 **Example 4: Construction of vectors to investigate the effects of glycosylation and
secretion, inclusion of Tat and inclusion of Gag (p17/24) on gp120 and gp120
fusions**

Vectors were constructed as shown in Figure 14 (schematic).

30

pRix 28 and pRix29 (Figures 7 and 8)

pRix28 and 29 containing ds gp120c NefTat^m and ds gp120c trNefTat were generated by transferring the AccI-BamHI fragments from pRix6 (2315bp) and pRix11 (2123bp) into similarly cut pRix12 (ds gp120c).

5 pRix30 and pRix31 (Figure 9)

To generate glycosylated and non-glycosylated fusion vectors of gp120c Nef without Tat, the NotI-KpnI fragment was transferred from pRix11 (1580bp) or pRix29 (1496bp) into similarly cut pRix15, a vector containing Tat/trNef.

10

pRix32

To generate the fusion containing p17/24, gp120 was PCR amplified from pgp120c using primers U1 and 3'120, p17/24-Nef was amplified from p73i-GN2 using primers
 15 5'120G and 3'Nef, and the two were PCR stitched using U1 and 3'Nef. p73I-GN2 contained a synthetic codon optimised sequence of p17/p24 based on the sequence of HXB2 (GenBank entry K03455) and designed using SynGene and assembled from overlapping oligonucleotides as described for codon optimised gp120 above, fused to HXB2 Nef, which had been obtained from plasmid pHXB□Pr (B.Maschera, E Furfine
 20 and E.D. Blair 1995 J.Virol 69 5431-5436) by PCR. Since the HXB2 nef gene in this plasmid contains a premature termination codon two overlapping PCRs were used to repair the codon (TGA [stop] to TGG [Trp]). The position of the repaired codon is underlined in the sequence. The p17/p24/Nef gene was inserted into the NotI and BamHI sites of plasmid p7313ie. The coding sequence and map is given in Figure 10.
 25 A * marks the p24/trNef junction.

p73I-GN2 contained a synthetic codon optimised sequence of p17/p24 based on the sequence of HXB2 (GenBank entry K03455) and designed using SynGene and assembled from overlapping oligonucleotides as described for codon optimised gp120
 30 above, fused to HXB2 Nef which had been obtained from plasmid pHXB□Pr (B.Maschera, E Furfine and E.D. Blair 1995 J.Virol 69 5431-5436) by PCR, and inserted into the NotI and BamHI sites of plasmid p7313ie.

Primers:

U1:

GAATTCGCGGCCGCAATGAAGGTCAAGGAGACCAGAAAGAACTACCAGC

5 ATCTGTG

3'120: TCTCTGCACGACGCGGCGCTTGGC

5'120G:

GCCAAGCGCCGCGTCGTGGAGAGAATGGGTGCCCCGAGCTTCGGTAC

3'Nef: GAATTCGGATCCTTAGCAGTTCTTGAAGTACTCCGG

10

Initial cycle:

94°C(30s) 20x[94°C (30s) 50°C (30s) 68°C (180s)] 68°C (120s) 4°C (0s)

Using pfx polymerase with 1x enhancer.

15 Stitch:

94°C(30s) 20x[94°C (30s) 50°C (30s) 72°C (180s)] 72°C (120s) 4°C (0s)

Using Vent polymerase in standard conditions + 2mM MgCl₂.

The product was cut with NotI and BamHI and cloned into p7313-ie.

20

On sequencing the construct was found to have a error in the signal peptide, which was corrected by transferring the 2560bp BstEII - KpnI fragment containing the back of gp120 to the front of Nef into pRix30.

25 pRix 33 (Figure 11), pRix34, and pRix35 (Figure 12)

The 2560bp BstEII - KpnI fragment containing the back of gp120 to the front of Nef was transferred to pRix31, pRix11 and pRix29 to make vectors pRix 33 (Figure 11), 34 and 35 (Figure 12) respectively.

30

pRix39 (gp120 codon optimised, minus secretion signal - p17/24 gag - Nef-Tat - Figure 13)

A fragment containing gag p17/24 was PCR amplified from vector pRix35 using primers:

5'120G:

5 GCCAAGCGCCGCGTCGTGGAGAGAATGGGTGCCCCGAGCTTCGGTAC

and

p24AS: CAACACTCTGGCTTTGTGTCC

Full length Nef was PCR amplified from pNTm using primers:

10 5'p24-N: GGACACAAAGCCAGAGTGTGATGGGCAAGTGGTCAAAAAGTAG

and

3'Nef: GAATTCGGATCCTTAGCAGTTCTTGAAGTACTCCGG

The two fragments were PCR stitched together using the end primers (5'120G and
15 3'Nef). The product was cut with Sall and KpnI, and the 423bp fragment containing
part of p24 and Nef was used to replace the corresponding fragment in pRix35 to
make pRix29 (Figure 13).

Results

20

Expression data is shown in Figure 14.

293T cell monolayers in 24 well plates were transfected with 1µg of each DNA
indicated using Lipofectamine 2000 in 0.6ml of Optimem. After 24 hours the cells
were detached and separated from the culture medium by centrifugation. Samples
25 equivalent to 1×10^4 cells or 12µl of medium were examined by PAGE and Western
blot.

The gp120c construct gave a highly glycosylated well secreted protein. Addition of c-
terminal Nef/Tat fusions (pRix 6 and pRix11) resulted in a reduction of the
30 intracellular protein levels and loss of secretion. Removal of the secretion signal from
gp120c (pRix12) gave a non-glycosylated non-secreted form of the protein.

As expected, fusion constructs with no secretion signal pRix28, 29, 31, 33 and 35, made non-glycosylated intracellular proteins in similar amounts, though expression from pRix35 was somewhat reduced. Surprisingly, when the secretion signal was present in constructs pRix30, 32 and 34, only pRix34 failed to be secreted. It appears that the presence of Tat in the fusion inhibits secretion of the protein. The initial pRix32.1 construct had a point mutation resulting in poor expression. This was corrected in pRix32.7, which showed greatly improved expression.

Refs:

- 10 Andre S. Seed B. Eberle J. Schraut W. Bultmann A. Haas J. (1998) Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *Journal of Virology*. 72(2):1497-503.

- 15 Vinner L. Nielsen HV. Bryder K. Corbet S. Nielsen C. Fomsgaard A. (1999) Gene gun DNA vaccination with Rev-independent synthetic HIV-1 gp160 envelope gene using mammalian codons. *Vaccine*. 17(17):2166-75

Collins KL. Baltimore D.(1999) HIV's evasion of the cellular immune response. *Immunological Reviews*. 168:65-74.

20

Example 5: Preparation of plasmid-coated 'gold slurry' for 'gene gun' DNA cartridges

- 25 Plasmid DNA (approximately 1µg/µl), eg. 100 ug, and 2µm gold particles, eg. 50 mg, (PowderJect), were suspended in 0.05M spermidine, eg. 100 ul, (Sigma). The DNA was precipitated on to the gold particles by addition of 1M CaCl₂, eg. 100ul (American Pharmaceutical Partners, Inc., USA). The DNA/gold complex was incubated for 10 minutes at room temperature, washed 3 times in absolute ethanol, eg.
- 30 3 x 1 ml, (previously dried on molecular sieve 3A (BDH)). Samples were resuspended in absolute ethanol containing 0.05mg/ml of polyvinylpyrrolidone (PVP, Sigma), and split into three equal aliquots in 1.5 ml microfuge tubes, (Eppendorf). The aliquots were for analysis of (a) 'gold slurry', (b) eluate- plasmid eluted from (a) and (c) for

preparation of gold/ plasmid coated Tefzel cartridges for the 'gene gun', (see Example 3 below). For preparation of samples (a) and (b), the tubes containing plasmid DNA / 'gold slurry' in ethanol / PVP were spun for 2 minutes at top speed in an Eppendorf 5418 microfuge, the supernatant was removed and the 'gold slurry' dried for 10 minutes at room temperature. Sample (a) was resuspended to 0.5 – 1.0 ug / ul of plasmid DNA in TE pH 8.0, assuming approx. 50 % coating. For elution, sample (b) was resuspended to 0.5 – 1.0 ug / ul of plasmid DNA in TE pH 8.0 and incubated at 37°C for 30 minutes, shaking vigorously, and then spun for 2 minutes at top speed in an Eppendorf 5418 microfuge and the supernatant, eluate, was removed and stored at -20°C. The exact DNA concentration eluted was determined by spectrophotometric quantitation using a Genequant II (Pharmacia Biotech).

Example 6: Preparation of Cartridges for DNA immunisation

Preparation of cartridges for the Accell gene transfer device was as previously described (Eisenbraun et al DNA and Cell Biology, 1993 Vol 12 No 9 pp 791-797; Pertner et al). Briefly, plasmid DNA was coated onto 2 µm gold particles (DeGussa Corp., South Plainfield, N.J., USA) and loaded into Tefzel tubing, which was subsequently cut into 1.27 cm lengths to serve as cartridges and stored desiccated at 4°C until use. In a typical vaccination, each cartridge contained 0.5 mg gold coated with a total of 0.5 µg DNA/cartridge.

Example 7: PMID immunisations including using gp120-Nef-Tat triple fusion lacking gp120 secretion signal

Protocol: For PMID immunisations (DNA) cartridges were prepared for using standard methods as described in Examples 5 and 6. A DNA loading rate of 2, which will give approximately 0.5 µg DNA/cartridge was used and each immunisation consisted of two shots. Balb/c mice were given a primary immunisation of DNA (using PMID). The mice were boosted 28 days later with DNA (using PMID). Mice were culled 7 days later and serum and spleens were collected. The splenocytes were

harvested by teasing out the spleen cells and erythrocytes were lysed. The splenocytes were washed and counted. Specialised ELISpot plates (coated with interferon-gamma capture antibody and blocked) were used. Splenocytes were transferred to these plates and incubated overnight at 37°C/5% CO₂ in the presence of a gp120 peptide. The
5 splenocytes were lysed and the plate developed using standard procedures to demonstrate the number of interferon-gamma secreting cells present. Serum was analysed by ELISA assay to detect for specific antibodies. Results are shown in Figures 14-18.

10 Conclusion

Unexpectedly, the cellular immune response of mice immunised with dsGP120 (gp120 lacking secretion signal) expressing constructs was approximately double that of mice immunised with gp120 constructs (see Figure 15 and 16). This was consistent with the observation that in *in vitro* transfection studies the expression of dsGP120 had
15 remained largely cell associated, whereas gp120 had been excreted.

Inclusion of Tat (mutated Tat) in the dsGP120 constructs increased the cellular immune response to twice that of the dsGP120 constructs without Tat (Figures 15 and 16). Tat on its own did not affect the immune response to gp120, but acted
20 synergistically with dsGP120 to optimise the cellular response.

CLAIMS

1. A polynucleotide which comprises a sequence encoding a gp120 molecule
5 which is non-glycosylated when expressed in a mammalian target cell, operably linked to a heterologous promoter.
2. The polynucleotide according to claim 1 wherein the gp120 molecule lacks a functional secretion signal.
10
3. The polynucleotide according to claim 1 or claim 2 wherein the gp120 molecule is expressed as a fusion protein comprising at least one other HIV protein.
4. The polynucleotide according to claim 3 wherein the gp120 encoding sequence
15 is linked to a Tat encoding sequence to encode a gp120 and Tat-containing fusion protein.
5. The polynucleotide according to claim 4 wherein the gp120 sequence is further linked to a Nef encoding sequence to encode a gp120, Tat and Nef-containing fusion
20 protein.
6. The polynucleotide according to claim 5 wherein the fusion is a gp120-Nef-Tat fusion.
- 25 7. The polynucleotide according to claim 5 wherein the gp120 sequence is further linked to a Gag encoding sequence.
8. The polynucleotide according to claim 7 wherein the fusion is a gp120-Gag-Nef-Tat fusion.
30
9. The polynucleotide according to claim 7 or claim 8 wherein the Gag comprises P17 and/or 24.

10. The polynucleotide according to any one of claims 1 to 9 wherein one or more of the sequences encoding gp120, Nef, Tat or Gag is or are codon optimised to resemble the codon usage in a highly expressed human gene.
- 5 11. A polynucleotide sequence selected from the group:
1. gp120 codon optimised, minus secretion signal
 2. gp120 codon optimised, minus secretion signal – tr Nef
 3. gp120 codon optimised, minus secretion signal – tr Nef – Tat
 - 10 4. gp120 codon optimised, minus secretion signal – Nef - Tat
 5. gp120 codon optimised, minus secretion signal – p17/24 Gag – tr Nef
 6. gp120 codon optimised, minus secretion signal – p17/24 Gag – tr Nef - Tat
 7. gp120 codon optimised, minus secretion signal - p17/24 gag - Nef-Tat
 8. gp120 codon optimised, minus secretion signal - p17/24 gag - mNef-Tat
 - 15 9. gp120 codon optimised, minus secretion signal - p17/24 gag - L1Nef-Tat
 10. gp120 codon optimised, minus secretion signal - p17/24 gag - L2Nef-Tat
 11. gp120 codon optimised, minus secretion signal - p17/24 gag - LLNef-Tat
 12. gp120 codon optimised, minus secretion signal - p17/24 gag - mLLNef-Tat
 13. gp120 codon optimised, minus secretion signal - p17/24 gag - mL1Nef-Tat
 - 20 14. gp120 codon optimised, minus secretion signal - p17/24 gag - mL2Nef-Tat
12. The polynucleotide according to any one of claims 1 to 11 wherein the promoter is the promoter from HCMV IE gene.
- 25 13. The polynucleotide according to claim 12 wherein the 5' end of the promoter comprises exon 1.
14. A vector comprising a polynucleotide as claimed in any one of claims 1 to 13.
- 30 15. The vector according to claim 14 which is a double stranded DNA plasmid.

16. A pharmaceutical composition comprising a nucleotide sequence according to any one of claims 1 to 13, a vector of claim 14 or 15 and a pharmaceutically acceptable excipient, diluent, carrier or adjuvant.

5 17. The pharmaceutical composition according to claim 16 wherein the carrier is a plurality of particles such as gold beads.

18. An intradermal delivery device comprising a pharmaceutical composition according to claim 16 or 17.

10

19. A method of treating a patient suffering from or susceptible to a disease comprising administering a safe and effective amount of a pharmaceutical composition according to claim 16 or 17.

15 20. A polynucleotide or a vector according to any one of claims 1 to 15 for use in medicine.

21. Use of a polynucleotide or a vector according to any one of claims 1 to 15 in the manufacture of a medicament for the treatment of disease.

20

22. A process for the production of a polynucleotide according to any one of claim 1 to 13 comprising linking a nucleotide sequence encoding a non-glycosylated gp120 sequence and optionally a sequence encoding an HIV regulatory protein, to a heterologous promoter sequence.

25

23. A polypeptide encoded by the polynucleotide or vector according to any one of claims 1 to 15.

24. A pharmaceutical composition comprising a polypeptide according to claim 23
30 and a pharmaceutically acceptable excipient, diluent, carrier or adjuvant.

25. A method of treating a patient suffering from or susceptible to a disease comprising administering a safe and effective amount of a pharmaceutical composition according to claim 24.
- 5 26. A polypeptide according to claim 23 for use in medicine.
27. Use of a polypeptide according to claim 23 in the manufacture of a medicament for the treatment of disease.

Figure 1

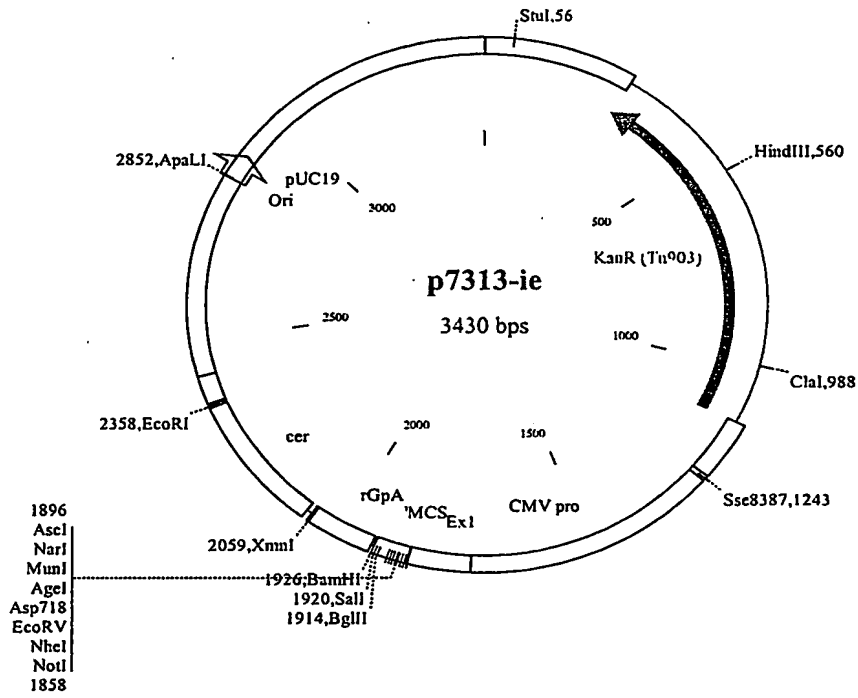


Figure 2

The amino acid sequence of the W61D gp120 is below. The signal sequence is underlined and bold, up to the predicted cleavage site between amino acids 29 and 30. This is the sequence removed in dsGP120 (pRix12 etc).

MKVKETRKNYQHLWRWGTMLLGMLMICSAAEQLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATH
ACVPTDPNPQEVVLGNVTEYFNMWKNNMVDQMHEDIISLWDQSLKPCVKLTPLCVTLDCDDVNTTNSTTTT
SNGWTGEIRKGEIKNCSFNITTSIRDKVQKEYALFYNLDVVPIDDDNATTKNKTTRNFRLIHCNSSVMTQA
CPKVSFEPIPIHYCAPAGFAILKCNNKTFDGKGLCTNVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVIRSD
NFMNTKTIIVQLNESVAINCTRPNNNTRKGIHIGPGRAFYAARKIIGDIRQAHCNLSRAQWNNLTKQIVI
KLREHFGNKTIFKNQSSGGDPEIVRHSFNCGGEFFYCDTTQLFNSTWNGTEGNNTTEGNSTITLPCRICKQII
NMWQEVGKAMYAPPIGGQIRCSSNITGLLLTRDGGTEGNGTENETEIFRPGGGDMRDNRSELYKYKVVKV
EPLGVAPTRAKRRVVQR

The codon optimised DNA sequence for the W61D gp120 gene is:

ATGAAGGTCAAGGAGACCAGAAAGAACTACCAGCATCTGTGGCGCTGGGGCACCATGCTCCTGGGAATGCT
GATGATCTGCTCCGCCCGGAGCAGCTGTGGGTACCGTCTACTACGGCGTGCTGTGTGGAAGGAGGCCA
CGACCACCCTCTTCTGCGCGAGCGACGCCAAGGCCTACGACACGGAAGTGCATAACGTGTGGGCGACGCAT
GCTTGCGTGCCTACGGACCCCAACCCCAAGGAGGTGGTGTCTGGGAAACGTGACCGAGTACTTCAACATGTG
GAAGAATAACATGGTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCAGTCCCTGAAGCCCTGCG
TGAAGCTGACGCCTCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACC
AGCAACGGCTGGACCGGAGAGATTGGAAGGGCGAGATCAAGAACTGCTCCTTCAATATCACGACCTCGAT
CAGAGACAAGGTGCAGAAGGAATACGCGCTGTTTTATAATCTCGATGTGGTCCCCATCGACGACGACAATG
CCACCACCAAGAACAAGACGACGCGTAATTTAGACTCATTCAGTCAACAGCAGCGTCATGACGCAGGCC
TGCCCCAAGGTGTCCTTCGAACCAATCCCGATCCATTACTGTGCCCCTGCCGGATTTCGCGATCCTCAAGTG
TAACAACAAGACCTTCGACGGGAAGGGCCTGTGCACCAACGTGACGACGGTGCAGTGCACCCATGGCATCC
GCCCCGTGCTGAGCACCCAGCTGCTGCTGAACGGGTCCCTGGCTGAGGAGGAGGTGGTGATCCGGTCGGAC
AACTTCATGGACAACACCAAGACAATCATCGTCCAGCTGAACGAGTCTGTGGCGATTAACTGTACCCGGCC
TAACAACAACACCCGTAAGGGCATCCACATCGGGCCTGGACGGGCCTTCTATGCCGCCCCGAAGATCATCG
GCGACATCCGGCAGGCCCATTGCAACCTCTCCCGCGCCAGTGGAATAACACCCTGAAGCAGATCGTGATC
AAGCTGAGAGAGCACTTTGGAACAAGACCATCAAGTTCAATCAGAGTTCTGGCGGAGACCCCGAGATCGT
GCGGCACTCCTTCAACTGCGGGGGCGAGTTCTTCTACTGCGATACGACACAGCTCTTCAACTCCACCTGGA
ACGGCACCGAGGGCAACAACACAGAGGGAACTCCACTATCACCTCCCTTGCCGCATCAAGCAGATCATC
AACATGTGGCAGGAGGTGGGAAAGGCCATGTATGCCCCCCCCATCGGGGGCCAGATCCGCTGCTCCTCCAA
CATCACCGGCCTGCTGCTCACCAGAGACGGGGGCACCGAGGGCAACGGCACGGAGAACGAGACGGAGATCT
TCAGGCCCGGCGGGCGGACATGAGGGATAACTGGCGGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGTG
GAGCCGCTCGGCGTGGCCCCCACCCGGGCCAAGCGCCGCTCGTGCAGAGATGA

Figure 3

Map of pRix15244:

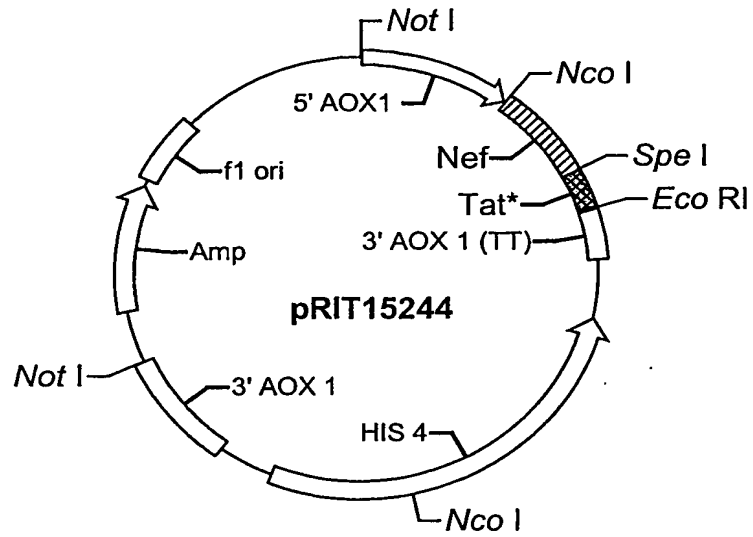
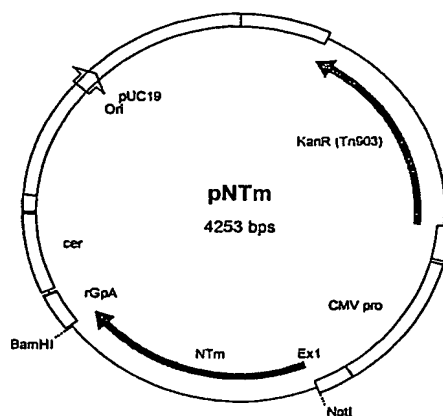


Figure 4

Plasmid pNTm:



Sequence of insert:

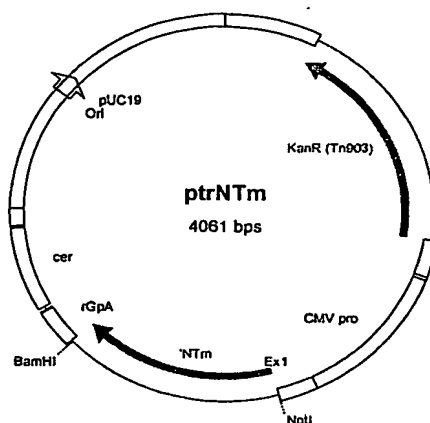
ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGA
 GCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACAAGTAGCAATA
 CAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACA
 CCTCAGGTACCTTTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGG
 GGGACTGGAAGGGCTAATCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAG
 GCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGATGGTGC
 TACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACA
 CCCTGTGAGCCTGCATGGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAG
 CATTTTCATCACGTGGCCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCT
 AGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAAGTCTTGTTACCAATTGCTATTGTAAAAGTG
 TTGCTTTTCATTGCCAAGTTTGTTCATAACAGCTGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGAC
 AGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCC
 AAAGGGGAGCCGACAGGCCCGAAGGAATAA

Amino acid sequence of antigen:

MGGKWSKSSVVGWPTVRERMRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQEE
 EEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYT
 PPGVRYPLTFGWICYKLVPEPDKVEEANKGENTSLHPVSLHGMDDPEREVLEWRFD SRLAFH
 HVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITAALGISYGRK
 KRRQRRRPPQGSQTHQVSLSKQPTSQSKGEPTGPKE

Figure 5

Plasmid ptrNTm:



Sequence of insert:

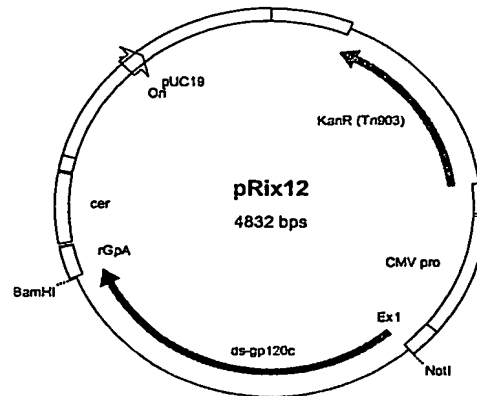
ATGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTAG
 CCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATTCACCTCCCAACGAAGACAAGATATCCTTGATC
 TGTGGATCTACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATAT
 CCACCTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGG
 AGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAGAGAGAAGTGTTAGAGT
 GGAGGTTTGACAGCCGCTAGCATTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGC
 ACTAGTGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAACTGCTTGTAC
 CAATTGCTATTGTAAAAAGTGTTGCTTTTCATTGCCAAGTTTGTTCATAACAGCTGCCTTAGGCATCTCCT
 ATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCA
 AAGCAACCCACCTCCCAATCCAAAGGGGAGCCGACAGGCCCGAAGGAATAA

Amino acid sequence of antigen:

MVGFVPTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPGVRY
 PLTFGWICYKLVPVEPKVEEANKGENTSLHHPVSLHGMDPEREVLEWRFD SRLAFHHVARELHPEYFKNC
 TSEPVDPRLEPWKHPGSQPKTACTNICYCKKCCFHCQVCFITAALGISYGRKKRRQRRRPPQGSQTHQVSL
 KQPTSQSKGEPTGPKE

Figure 6

Plasmid pRix12:



Sequence of insert:

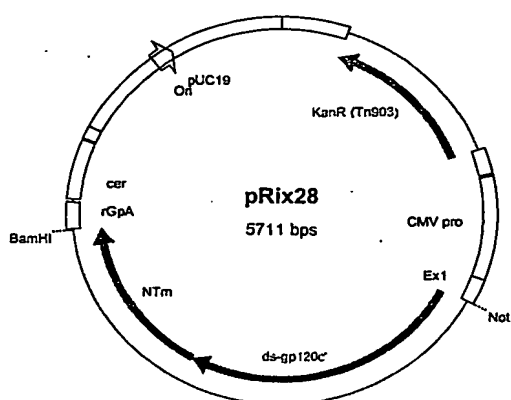
ATGGCCGAGCAGCTGTGGGTCACCGTCTACTACGGCGTGCTGTGTGGAAGGAGGCCACGACCACCTCTT
 CTGCGCGAGCGACGCCAAGGCCTACGACACGGAAAGTGCATAACGTGTGGGCGACGCATGCTTGCGTGCCTA
 CGGACCCCAACCCCCAGGAGGTGGTGTCTGGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
 GTGGATCAGATGCACGAGGACATCATCTCTCTGTGGGACCAAGTCCCTGAAGCCCTGCGTGAAGCTGACGCC
 TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACCAGCAACGGCTGGA
 CCGGAGAGATTTCGGAAGGGCGAGATCAAGAAGTCTCCTTCAATATCACGACCTCGATCAGAGACAAGGTG
 CAGAAGGAATACGCGCTGTTTTATAATCTCGATGTGGTCCCCATCGACGACGACAATGCCACCACCAAGAA
 CAAGACGACGCGTAATTTTCAGACTCATTCACTGCAACAGCAGCGTCATGACGCGAGGCCTGCCCAAGGTGT
 CCTTCGAACCAATCCCGATCCATTACTGTGCCCCCTGCCGGATTTCGCGATCCTCAAGTGTAAACAAGACC
 TTCGACGGGAAGGGCCTGTGCACCAACGTGACGACGGTGCAGTGCACCCATGGCATCCGCCCCGTCTGTGAG
 CACCCAGCTGCTGCTGAACGGGTCCCTGGCTGAGGAGGAGGTGGTGATCCGGTCCGGACAACCTTCATGGACA
 ACACCAAGACAATCATCGTCCAGCTGAACGAGTCTGTGGCGATTAACTGTACCCGGCCTAACAAACAACACC
 CGTAAGGGCATCCACATCGGGCCTGGACGGGCCTTCTATGCCGCCCGCAAGATCATCGGCGACATCCGGCA
 GGCCCATTTGCAACCTCTCCCGCGCCAGTGAATAACACCCCTGAAGCAGATCGTGATCAAGCTGAGAGAGC
 ACTTTGGAAACAAGACCATCAAGTTCAATCAGAGTTCTGGCGGAGACCCCGAGATCGTGCGGCACTCCTTC
 AACTGCGGGGGCGAGTTCTTCTACTGCGATACGACACAGCTCTTCAACTCCACCTGGAACGGCACCGAGGG
 CAACAACACAGAGGGAACTCCACTATCACCTCCCTTGCCGCATCAAGCAGATCATCAACATGTGGCAGG
 AGGTGGGAAAGGCCATGTATGCCCCCCCCATCGGGGGCCAGATCCGCTGCTCCTCCAACATCACCGGCCTG
 CTGCTCACCAGAGACGGGGGCACCGAGGGCAACGGCACGGAGAACGAGACGGAGATCTTCAGGCCCGGCGG
 CGGCGACATGAGGGATAACTGGCGGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGTGGAGCCGCTCGGCG
 TGGCCCCACCCGGGCCAAGCGCCGCGTCTGTGAGAGATGA

Amino acid sequence of antigen:

MAEQLWVTVYYGVVPVWKEATTTLFCASDAKAYDTEVHNWATHACVPTDPNPQEVVLGNVTEYFNMWKNNM
 VDQMHEDIISLWDQSLKPCVKLTPLCVTLDCDDVNTTNTSTTTTNSGWTGEIRKGEIKNCSFNITTSIRDKV
 QKEYALFYNLDVVPIDDDNATTKNKTTRNFRLIHCNSSVMTQACPKVSFEPIPIHYCAPAGFAILKCNKNT
 FDGKGLCTNVSTVQCTHGIRPVVSTQLLLNGSLAEVVRSDNFMDNTKTIIVQLNESVAINCTRPNNNT
 RKGIIHIGPGRAFYAARKIIGDIRQAHCNLSRAQWNNTLKQIVIKLREHFGNKTIKFNQSSGGDPEIVRHSF
 NCGGEFFYCDTTQLFNSTWNGTEGNNTGNSTITLPCRIKQIINMWQEVGKAMYAPPIGGQIRCSSNITGL
 LLTRDGGTEGNGTENETEIFRPGGGDMRDNRSELYKYKVVKVEPLGVAPTRAKRRVVQR

Figure 7

Plasmid pRix28:



Sequence of insert:

ATGGCCGAGCAGCTGTGGGTCACCGTCTACTACGGCGTGCTGTGTGGAAGGAGGCCACGACCACCCTCTT
 CTGCGCGAGCGACGCCAAGGCCTACGACACGGAAGTGCATAACGTGTGGGCGACGCATGCTTGCGTGCCCTA
 CGGACCCCAACCCCAAGGAGGTGGTGGTGGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
 GTGGATCAGATGCACGAGGACATCATCTCTGTGGGACCAGTCCCTGAAGCCCTGCGTGAAGCTGACGCC
 TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACCAGCAACGGCTGGA
 CCGGAGAGATTTCGAAGGGCGAGATCAAGAACTGCTCCTTCAATATCACGACCTCGATCAGAGACAAGGTG
 CAGAAGGAATACGCGCTGTTTATAATCTCGATGTGGTCCCATCGACGACGACAATGCCACCACCAAGAA
 CAAGACGACGCGTAATTCAGACTCATTCACTGCAACAGCAGCGTCATGACGCAAGCCCTGCCCAAGGTGT
 CCTTCGAACCAATCCCGATCCATTACTGTGCCCTGCGGATTTCGCGATCCTCAAGTGTAAACAACAAGACC
 TTCGACGGGAAGGGCCTGTGCACCAACGTCAGCAGGTCAGTGCACCCATGGCATCCGCCCGCTCGTGAG
 CACCCAGCTGCTGCTGAACGGGTCCCTGGCTGAGGAGGAGTGGTGATCCGGTCGGACAACCTTCATGGACA
 ACACCAAGACAATCATCGTCCAGCTGAACGAGTCTGTGGCGATTAACTGTACCCGGCCCTAACACAACACAC
 CGTAAGGGCATCCACATCGGGCCTGGACGGGCCCTTCTATGCCGCCCCGCAAGATCATCGGCGACATCCGGCA
 GGCCATTGCAACCTCTCCCGCGCCAGTGGAAATAACACCCTGAAGCAGATCGTGATCAAGCTGAGAGAGC
 ACTTTGGAACAAGACCATCAAGTCAATCAGAGTCTGGCGGAGACCCGAGATCGTGCGGCACCTCCTTC
 AACTGCGGGGGCGAGTTCTTCTACTGCGATACGACACAGCTCTTCAACTCCACCTGGAACGGCACCGAGGG
 CAACAACACAGAGGGGAACTCCACTATCACCTCCCTTGCCGCATCAAGCAGATCATCAACATGTGGCAGG
 AGGTGGGAAAGGCCATGTATGCCCCCCCCATCGGGGGCCAGATCCGCTGCTCCTCCAACATCACCGGCCTG
 CTGCTCACCAGAGACGGGGGCACCGAGGGCAACGGCACGGAGAACGAGACGGAGATCTTCAGGCCCGGCGG
 CGGCGACATGAGGGATAACTGGCGGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGTGGAGCCGCTCGGCG
 TGGCCCCCACCCGGGCCAAGCGCCGCGTCTGTGCAGAGAATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTT
 GGATGGCCTACTGTAAAGGAAAGAATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCG
 AGACCTGGAACCAATGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCCTGGCTAG
 AAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG
 GCAGCTGTAGATCTTAGCCACTTTTTAAAGAAAAGGGGGGACTGGAAGGGCTAATCACTCCCAACGAAG
 ACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAG
 GGCCAGGGGTCAGATATCCACTGACC'TTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTA
 GAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGA
 GAGAGAAGTGTAGAGTGGAGGTTTGACAGCCGCTAGCATTTTCATCACGTGGCCCGAGAGCTGCATCCGG
 AGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAG

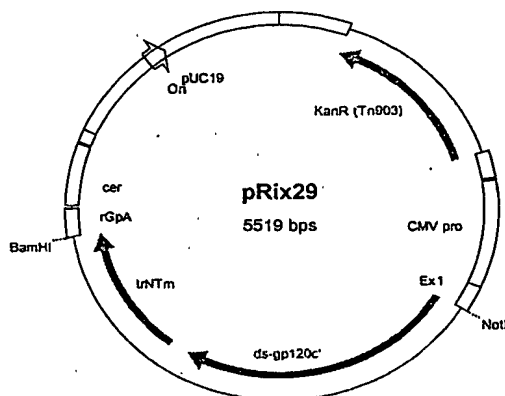
CCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTGCTTTCATTGCCAAGTTTGTTTCATAACAGC
TGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTC
ATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCAAAGGGGAGCCGACAGGCCCGAAGGAATAA

Amino acid sequence of antigen:

MAEQLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLGNVTEYFNMWKNNM
VDQMHEDIISLWDQSLKPCVKLTPLCVTLDCDDVNTTNSTTTTSSNGWTGEIRKGEIKNCSFNITTSIRDKV
QKEYALFYNLDVVPIDDDNATTKNKTTRNFRLLHCNSSVMTQACPKVSFEPIPIHYCAPAGFAILKCNNKT
FDGKGLCTNVSTVQCTHGIRPVVSTQLLNGLSLAESEVIRSDNFMDNTKTIIVQLNESVAINCTRPNNNT
RKGIIHIGPGRAFYAARKIIGDIRQAHCNLSRAQWNNTLKQIVIKLREHFGNKTIKFNQSSGGDPEIVRHSF
NCGGEFFYCDTTQLFNSTWNGTEGNNTEGNSTITLPCRIKQIINMWQEVGKAMYAPPIGGQIRCSSNITGL
LLTRDGGTEGNGTENETEIFRPGGDMRDNRSELYKYKVVKVEPLGVAPTRAKRRVVQRMGGKWSKSSVV
GWPTVRERMRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQEEEEVGFPVTPQVPLRPMTYK
AAVDLSHFLKEKGGLEGLIHSQRRQDILDWLIIYHTQGYFPDWQNYTPGPGVRYPLTFGWICYKLVPEPDKV
EEANKGENTSLHPVSLHGMDDPEREVLWRFD SRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQ
PKTACTNCYCKKCCFHCQVCFITAALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSKGEPTGPKE

Figure 8

Plasmid pRix29:



Sequence of insert:

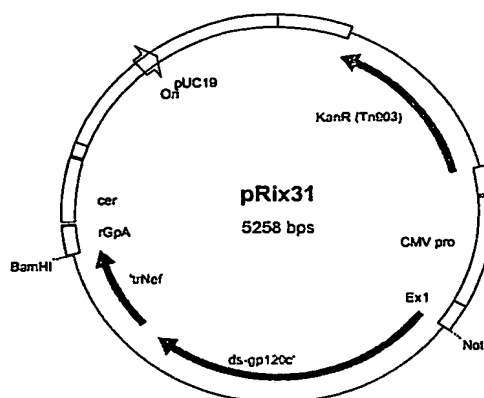
ATGGCCGAGCAGCTGTGGGTCAACGCTCTACTACGGCGTGCCTGTGTGGAAGGAGGCCACGACCACCCTCTT
 CTGCGCGAGCGACGCCAAGGCCTACGACACGGAGTGCATAACGTGTGGGCGACGCATGCTTGCGTGCCTA
 CGGACCCCAACCCCCAGGAGGTGGTGTCTGGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
 GTGGATCAGATGCACGAGGACATCATCTCTGTGTGGGACCAGTCCCTGAAGCCCTGCGTGAAGCTGACGCC
 TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACCAGCAACGGCTGGA
 CCGGAGAGATTTCGGAAGGGCGAGATCAAGAAGTGTCTCCTTCAATATCAGGACCTCGATCAGAGACAAGGTG
 CAGAAGGAATACGCGCTGTTTTATAATCTCGATGTGGTCCCCATCGACGACGACAATGCCACCACCAAGAA
 CAAGACGACGCGTAATTTAGACTCATTCAGTCAACAGCAGCGTCATGACGACGGCCTGCCCAAGGTGT
 CCTTCGAACCAATCCCGATCCATTACTGTGCCCCGTGCCGGATTTCGCGATCCTCAAGTGTAAACAAGACC
 TTCGACGGGAAGGGCCTGTGCACCAACGTGACGACGGTGCAGTGCACCCATGGCATCCGCCCCGTCGTGAG
 CACCCAGCTGCTGCTGAACGGGTCCCTGGCTGAGGAGGAGGTGGTGATCCGGTCCGGACAACCTTCATGGACA
 ACACCAAGACAATCATCGTCCAGCTGAACGAGTCTGTGGCGATTAACTGTACCCGGCCCTAACAAACACACC
 CGTAAGGGCATCCACATCGGGCCTGGACGGGCCTTCTATGCCCGCCGCAAGATCATCGGCGACATCCGGCA
 GGCCCATTGCAACCTCTCCCGCGCCAGTGGAATAACACCCCTGAAGCAGATCGTGATCAAGCTGAGAGAGC
 ACTTTGGAACAAGACCATCAAGTTCAATCAGAGTTCTGGCGGAGACCCCGAGATCGTGCGGCACCTCCTTC
 AACTGCGGGGGCGAGTTCTTCTACTGCGATACGACACAGCTCTTCAACTCCACCTGGAACGGCACCCGAGGG
 CAACAACACAGAGGGAACTCCACTATCACCTCCCTTGCCGCATCAAGCAGATCATCAACATGTGGCAGG
 AGGTGGGAAAGGCCATGTATGCCCCCCCATCGGGGGCCAGATCCGCTGCTCCTCCAACATCACCGGCCTG
 CTGCTCACAGAGACGGGGGCACCGAGGGCAACGGCACGGAGAACGAGACGGAGATCTTCAGGCCCGGCGG
 CGGCGACATGAGGGATAACTGGCGGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGTGGAGCCGCTCGGCG
 TGGCCCCCACC CGGGCCAAGCGCCGCTCGTGACAGAGAATGGTGGGTTTTCCAGTCACACCTCAGGTACCT
 TTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAGAAAAGGGGGGACTGGAAGG
 GCTAATTCACCTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTG
 ATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTA
 CCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCTGTGAGCCT
 GCATGGAATGGATGACCCTGAGAGAGAAGTGTAGAGTGGAGGTTTGACAGCCGCTAGCATTTTCATCACG
 TGGCCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCAGTGTAGCCAGTAGATCCTAGACTAGAGCCC
 TGGAAGCATCCAGGAAGTCAGCCTAAACTGCTTGTACCAATTGCTATTGTAAAAGTGTGCTTTTCATTG
 CCAAGTTTGTTCATAACAGCTGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGAC
 CTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCAAAGGGGAGCCG
 ACAGGCCCGAAGGAATAA

Amino acid sequence of antigen:

MAEQLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNWATHACVPTDPNPQEVVLGNVTEYFNMWKNNM
VDQMHEDIISLWDQSLKPCVKLTPLCVTLDCDDVNTTNSTTTTSNGWTGEIRKGEIKNCSFNITTSIRDKV
QKEYALFYNLDVVPIDDDNATTKNKTTRNFRLIHCNSSVMTQACPKVSFEPIPIHYCAPAGFAILKCNNKT
FDGKGLCTNVSTVQCTHGIRPVVSTQLLLNGSLAEDEVIRSDNFMDNTKTIIVQLNESVAINCTRPNNNT
RKGIHIGPGRAFYAARKIIGDIRQAHCNLSRAQWNNTLKQIVIKLREHFGNKTIKFNQSSGGDPEIVRHSF
NCGGEFFYCDTTQLFNSTWNGTEGNNTTEGNSTITLPCRIKQIINMWQEVGKAMYAPPIGGQIRCSSNITGL
LLTRDGGTEGNGTENETEIFRPGGGDMRDNRSELYKYKVVKVEPLGVAPTRAKRRVVQRMVGFVPTPQVP
LRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDWIYHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLV
PVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFD SRLAFHHVARELHPEYFKNCTSEPVDPRLEP
WKHPGSQPKTACTNCYCKKCCFHCQVCFITAALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSKGEP
TGPKE

Figure 9

Plasmid pRix31:



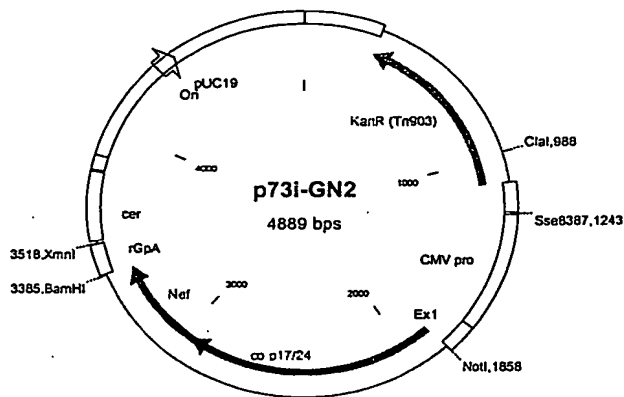
Sequence of insert:

ATGGCCGAGCAGCTGTGGGTCAACGTCTACTACGGCGTGCCGTGTGTGGAAGGAGGCCACGACCACCCCTCTT
 CTGCGCGAGCGACGCCAAGGCCTACGACACGGAAGTGCATAACGTGTGGGCGACGCATGCTTGCGTGCCTA
 CGGACCCCAACCCCAAGGAGGTGGTGTGGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
 GTGGATCAGATGCACGAGGACATCATCTCTCTGTGGGACCAGTCCCTGAAGCCCTGCGTGAAGCTGACGCC
 TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACCAGCAACGGCTGGA
 CCGGAGAGATTTCGAAGGGCGAGATCAAGAACTGCTCCTTCAATATCAGACCTCGATCAGAGACAAGGTG
 CAGAAGGAATACGCGCTGTTTTATAATCTCGATGTGGTCCCCATCGACGACGACAATGCCACCACCAAGAA
 CAAGACGACGCGTAATTTCACTCATTCACTGCAACAGCAGCGTCATGACGACGGCCTGCCCCAAGGTGT
 CCTTCGAACCAATCCCGATCCATTACTGTGCCCCTGCCGGATTTCGCGATCCTCAAGTGTAACAACAAGACC
 TTCGACGGGAAGGGCCTGTGCACCAACGTCAAGCAGCGTGCAGTGCACCCATGGCATCCGCCCCGTCGTGAG
 CACCCAGCTGCTGCTGAACGGGTCCCTGGCTGAGGAGGAGGTGGTGATCCGGTCGGACAACCTTCATGGACA
 ACACCAAGACAATCATCGTCCAGCTGAACGAGTCTGTGGCGATTAACTGTACCCGGCCTAACACAACACC
 CGTAAGGGCATCCACATCGGGCCTGGACGGGCCTTCTATGCCGCCCGCAAGATCATCGGCGACATCCGGCA
 GGCCATTGCAACCTCTCCCGCGCCAGTGGAATAACACCCTGAAGCAGATCGTGATCAAGCTGAGAGAGC
 ACTTTGGAACAAGACCATCAAGTTCAATCAGAGTTCTGGCGGAGACCCGAGATCGTGCGGCACCTCCTTC
 AACTGCGGGGGCGAGTTCTTCTACTGCGATACGACACAGCTCTTCAACTCCACCTGGAACGGCACCAGGGG
 CAACAACACAGAGGGAACTCCACTATCACCTCCCTTGCCGCATCAAGCAGATCATCAACATGTGGCAGG
 AGGTGGGAAAGGCCATGTATGCCCCCCCCATCGGGGGCCAGATCCGCTGCTCCTCCAACATCACCGGCCTG
 CTGCTCACCAGAGACGGGGGCACCGAGGGCAACGGGCACGGAGAACGAGACGGAGATCTTCAGGCCCGGCGG
 CGGCGACATGAGGGATAACTGGCGGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGTGGAGCCGCTCGGCG
 TGGCCCCCACC CGGGCCAAGCGCCGCTCGTGACAGAGAATGGTGGGTTTTCCAGTCACACCTCAGGTACCT
 TTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTTAAAGAAAAGGGGGGACTGGAAGG
 GCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTG
 ATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTA
 CCAGTTGAGCCAGATAAGGTAGAAGAGGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCT
 GCATGGAATGGATGACCTGAGAGAGAAGTGTAGAGTGGAGGTTTGACAGCCGCTAGCATTTTCATCAG
 TGGCCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCTAA

Amino acid sequence of antigen:

MAEQLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNWATHACVPTDPNPQEVVLGNVTEYFNMWKNNM
VDQMHEDIISLWDQSLKPCVKLTPLCVTLDCDDVNTTNSTTTTSNGWTGEIRKGEIKNCSFNITTSIRDKV
QKEYALFYNLDVVPIDDDNATTKNKTTRNFRLIHCNSSVMTQACPKVSFEPIPIHYCAPAGFAILKCNNKT
FDGKGLCTNVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVIRSDNFMDNTKTIIVQLNESVAINCTRPNNNT
RKGIHIGPGRAFYAARKIIGDIRQAHCNLSRAQWNNTLKQIVIKLREHFGNKTIKFNQSSGGDPEIVRHSF
NCGGEFFYCDTTQLFNSTWNGTEGNNTTEGNSTITLPCRIKQIINMWQEVGKAMYAPPIGGQIRCSSNITGL
LLTRDGGTEGNGTENETEIFRPGGGDMRDNRSELYKYKVVKVEPLGVAPTRAKRRVVQRMVGFPVTPQVP
LRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLV
PVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFD SRLAFHHVARELHPEYFKNC

Figure 10

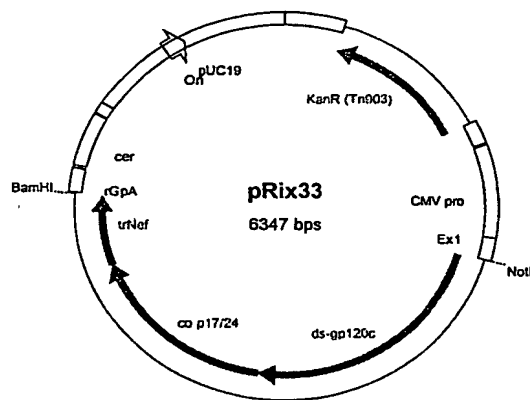


Sequence of insert:

TGGGTGCCCCGAGCTTCGGTACTGTCTGGTGGAGAGCTGGACAGATGGGAGAAAATTAGGCTGCGCCCGGGA
 GGCAAAAAGAAATACAAGCTCAAGCATATCGTGTGGGCCTCGAGGGAGCTTGAACGGTTTGCCGTGAACCC
 AGGCCTGCTGGAAACATCTGAGGGATGTCGCCAGATCCTGGGGCAATTGCAGCCATCCCTCCAGACCGGGA
 GTGAAGAGCTGAGGTCCTTGTATAACACAGTGGCTACCCTCTACTGCGTACACCAGAGGATCGAGATTAAG
 GATACCAAGGAGGCCTTGGACAAAATTGAGGAGGAGCAAAACAAGAGCAAGAAGAAGGCCAGCAGGCAGC
 TGCTGACACTGGGCATAGCAACCAGGTATCACAGAACTATCCTATTGTCCAAAACATTCAGGGCCAGATGG
 TTCATCAGGCCATCAGCCCCCGGACGCTCAATGCCTGGGTGAAGGTTGTCGAAGAGAAGGCCTTTTCTCCT
 GAGGTTATCCCCATGTTCTCCGCTTTGAGTGAGGGGGCCACTCCTCAGGACCTCAATACAATGCTTAATAC
 CGTGGGCGGCCATCAGGCCGCCATGCAAATGTTGAAGGAGACTATCAACGAGGAGGCAGCCGAGTGGGACA
 GAGTGCATCCCGTCCACGCTGGCCCAATCGCGCCCGGACAGATGCGGGAGCCTCGCGGCTCTGACATTGCC
 GGCACCACCTCTACACTGCAAGAGCAAATCGGATGGATGACCAACAATCCTCCCATCCCAGTTGGAGAAAT
 CTATAAACGGTGGATCATTCTCGGTCTCAATAAAATTGTTAGAATGTACTCTCCGACATCCATCCTTGACA
 TTAGACAGGGACCCAAAGAGCCTTTTAGGGATTACGTCGACCGGTTTTATAAGACCCTGCGAGCAGAGCAG
 GCCTCTCAGGAGGTCAAAAACCTGGATGACGGAGACACTCCTGGTACAGAACGCTAACCCCGACTGCAAAAC
 AATCTTGAAGGCACTAGGCCCGGCTGCCACCCTGGAAGAGATGATGACCGCCTGTCAGGGAGTAGGCGGAC
 CCGGACACAAAGCCAGAGTGTG*ATGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGAC
 TTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAGAAAAGGGGGGACTGGAAGGGCTAATTCACCTCCC
 AAAGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATTGGCAGAACTAC
 ACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGA
 TAAGGTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGGATGGATG
 ACCCGGAGAGAGAAGTGTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTTCATCACGTGGCCCGAGAGCTG
 CATCCGGAGTACTTCAAGAAGCTGCTGA

Figure 11

Plasmid pRix33:



Sequence of insert:

ATGGCCGAGCAGCTGTGGGTACCGTCTACTACGGCGTGCCGTGTGTGGAAGGAGGCCACGACCACCCTCTT
 CTGCGCGAGCGACGCCAAGGCCTACGACACGGAAGTGCATAACGTGTGGGCGACGCATGCTTGCGTGCCTA
 CGGACCCCAACCCCCAGGAGGTGGTGCTGGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
 GTGGATCAGATGCACGAGGACATCATCTCTCTGTGGGACCAGTCCCTGAAGCCCTGCGTGAAGCTGACGCC
 TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACCAGCAACGGCTGGA
 CCGGAGAGATTCCGAAGGGCGAGATCAAGAACTGCTCCTTCAATATCACGACCTCGATCAGAGACAAGGTG
 CAGAAGGAATACGCGCTGTTTTATAATCTCGATGTGGTCCCCATCGACGACGACAATGCCACCACCAAGAA
 CAAGACGACGCGTAATTTAGACTCATTCACTGCAACAGCAGCGTCATGACGCAGGCCTGCCCAAGGTGT
 CCTTCGAACCAATCCCGATCCATTACTGTGCCCTGCCGGATTTCGCGATCCTCAAGTGTAAACAACAAGACC
 TTCGACGGGAAGGGCCTGTGCACCAACGTGACGACGGTGCAGTGCACCCATGGCATCCGCCCCGTGCGTAG
 CACCCAGCTGCTGCTGAACGGGTCCCTGGCTGAGGAGGAGGTGGTGATCCGGTCCGACAACTTCATGGACA
 ACACCAAGACAATCATCGTCCAGCTGAACGAGTCTGTGGCGATTAACTGTACCCGGCCTAACAAACAACACC
 CGTAAGGGCATCCACATCGGGCCTGGACGGGCCTTCTATGCCGCCCGCAAGATCATCGGCGACATCCGGCA
 GGCCCATTGCAACCTCTCCCGCGCCCGAGTGGGAATAACACCCTGAAGCAGATCGTGATCAAGCTGAGAGAGC
 ACTTTGGAACAAGACCATCAAGTTCAATCAGAGTTCTGGCGGAGACCCGAGATCGTGCGGCACTCCTTC
 AACTGCGGGGGCGAGTTCTTCTACTGCGATACGACACAGCTCTTCAACTCCACCTGGAACGGCACCAGAGGG
 CAACAACACAGAGGGAACTCCACTATCACCTCCCTTGCCGCATCAAGCAGATCATCAACATGTGGCAGG
 AGGTGGGAAGGCCATGTATGCCCCCCCATCGGGGGCCAGATCCGCTGCTCCTCCAACATCACCGGCCTG
 CTGCTCACCAGAGACGGGGGCACCGAGGGCAACGGCAGGAGAACGAGACGGAGATCTTCAGGCCCGGCGG
 CGGCGACATGAGGGATAACTGGCGGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGTGGAGCCGCTCGGCG
 TGGCCCCCACC CGGGCCAAGCGCCGCTCGTGACAGAGAATGGGTGCCGAGCTTCGGTACTGTCTGGTGGG
 GAGCTGGACAGATGGGAGAAAATTAGGCTGCGCCCGGGAGGCAAAAAGAAATACAAGCTCAAGCATATCGT
 GTGGGCCTCGAGGGAGCTTGAACGGTTTGCCGTGAACCCAGGCCTGCTGGAAACATCTGAGGGATGTCGCC
 AGATCCTGGGGCAATTGCAGCCATCCCTCCAGACCGGGAGTGAAGAGCTGAGGTCCTTGTATAACACAGTG

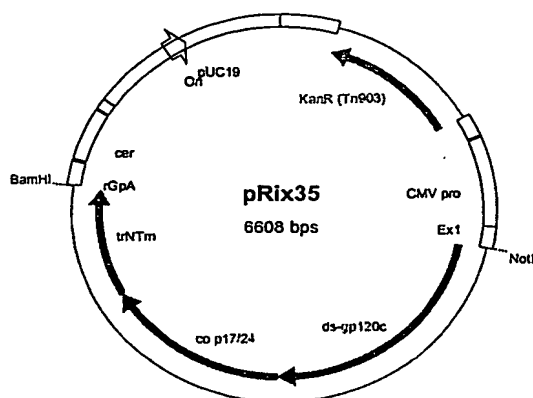
GCTACCCTCTACTGCGTACACCAGAGGATCGAGATTAAGGATACCAAGGAGGCCTTGGACAAAATTGAGGA
 GGAGCAAAACAAGAGCAAGAAGAAGGCCAGCAGGCAGCTGCTGACACTGGGCATAGCAACCAGGTATCAC
 AGAACTATCCTATTGTCCAAAACATTTCAGGGCCAGATGGTTTCATCAGGCCATCAGCCCCGGACGCTCAAT
 GCCTGGGTGAAGGTTGTGGAAGAGAAGGCCTTTTCTCCTGAGGTTATCCCATGTTCTCCGCTTTGAGTGA
 GGGGGCCACTCCTCAGGACCTCAATACAATGCTTAATACCGTGGGCGGCCATCAGGCCGCCATGCAAATGT
 TGAAGGAGACTATCAACGAGGAGGCAGCCGAGTGGGACAGAGTGCATCCCGTCCACGCTGGCCCAATCGCG
 CCCGGACAGATGCGGGAGCCTCGCGGCTCTGACATTGCCGGCACCACCTCTACACTGCAAGAGCAAATCGG
 ATGGATGACCAACAATCCTCCCATCCCAGTTGGAGAAATCTATAAACGGTGGATCATTCTCGGTCTCAATA
 AAATTGTTAGAATGTACTCTCCGACATCCATCCTTGACATTAGACAGGGACCCAAAGAGCCTTTTAGGGAT
 TACGTCGACCGGTTTTATAAGACCCTGCGAGCAGAGCAGGCCTCTCAGGAGGTCAAAAACCTGGATGACGGA
 GACACTCCTGGTACAGAACGCTAACCCCGACTGCAAAACAATCTTGAAGGCACTAGGCCCGGCTGCCACCC
 TGGAAGAGATGATGACCGCCTGTCAGGGAGTAGGCGGACCCGGACACAAAGCCAGAGTGTGATGGTGGGT
 TTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTT
 AAAAGAAAAGGGGGGACTGGAAGGGCTAATTCACCTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCT
 ACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTGAGATATCCACTGACC
 TTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGAGAGAACAC
 CAGCTTGTACACCTGTGAGCCTGCATGGAATGGATGACCCTGAGAGAGAAGTGTAGAGTGGAGGTTTG
 ACAGCCGCCTAGCATTTTCATCAGCTGGCCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCTAA

Amino acid sequence of antigen:

MAEQLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNWATHACVPTDPNPQEVVLGNVTEYFNMWKNM
 VDQMHEDIISLWDQSLKPCVKLTPLCVTLDCDDVNTTNSTTTTNSNGWTGEIRKGEIKNCSFNITTSIRDKV
 QKEYALFYNLDVVPIDDDNATTKNKTTRNFRLIHCNSSVMTQACPKVSFEPIPIHYCAPAGFAILKCNKKT
 FDGKGLCTNVSTVQCTHGIRPVVSTQLLLNGSLAEDEVIRSDNFMNTKTIIVQLNESVAINCTRPNNNT
 RKGIIHGPGRAFYAARKIIGDIRQAHCNLSRAQWNNTLKQIVIKLREHFGNKTIKFNQSSGGDPEIVRHSF
 NCGGEFFYCDTTQLFNSTWNGTEGNNTTEGNSTITLPCRKIQIINMWQEVGKAMYAPPIGGQIRCSSNITGL
 LLTRDGGTEGNGTENETEIFRPGGDMRDNRSELYKYKVVKVEPLGVAPTRAKRRVVQRMGARASVLSGG
 ELDRWEKIRLRPGGKKKYKLKHIVWASRELERFAVNPGLLETSEGCRQILGQLQPSLQTSSEELRSLYNTV
 ATLYCVHQRIEIKDTKEALDKIEEEQNKSKKKAQQAADTGHSNQVSQNYPIVQNIQGQMVHQAI SPRTL N
 AWWKVVEEKAFSPFVPMFSALSEGATPQDLNMTLNTVGGHQAAMQMLKETINEEAAEWDRVHPVHAGPIA
 PGQMREPRGSDIAGTTSTLQEQIGWMTNPPPIPVGEIYKRWIILGLNKIVRMYSPTSILDIRQGPKEPFRD
 YVDRFYKTLRAEQASQEVKNWMTETLLVQNPANPDCKTILKALGPAATLEEMMTACQGVGGPGHKARVLMVG
 FVTPQVPLRPMTYKAAVDLSHFLKEKGGLIHSQRRQDILDWLYHTQGYFPDWQNYTPGPGVRYPLT
 FGWCYKLVPEPDKVEEANKGENTSLLLHPVSLHGMDDPEREVLEWRFD SRLAFHHVARELHPEYFKNC

Figure 12

Plasmid pRix35:



Sequence of insert

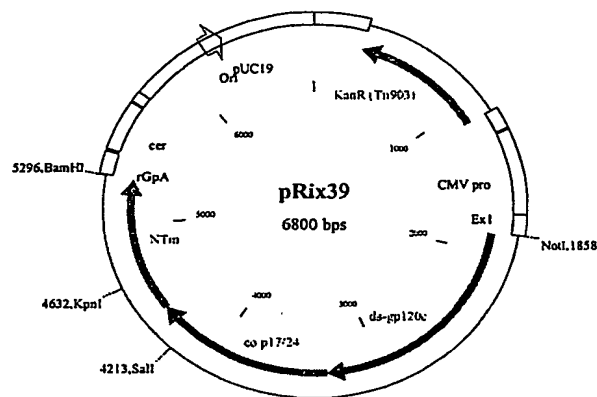
ATGGCCGAGCAGCTGTGGGTACCGTCTACTACGGCGTGCTGTGTGGAAGGAGGCCACGACCACCTCTT
 CTGCGCGAGCGACGCCAAGGCCTACGACACGGAAGTGCATAACGTGTGGGCGACGCATGCTTGGCTGCCTA
 CGGACCCCAACCCCCAGGAGGTGGTGTCTGGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
 GTGGATCAGATGCACGAGGACATCATCTCTGTGTGGGACCAGTCCCTGAAGCCCTGCGTGAAGCTGACGCC
 TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACCAGCAACGGCTGGA
 CCGGAGAGATTTCGAAGGGCGAGATCAAGAACTGCTCCTTCAATATCACGACCTCGATCAGAGACAAGGTG
 CAGAAGGAATACGCGCTGTTTTATAATCTCGATGTGGTCCCCATCGACGACGACAATGCCACCACCAAGAA
 CAAGACGACGCGTAATTTAGACTCATTCACTGCAACAGCAGCGTCATGACGCAGGCCTGCCCAAGGTGT
 CCTTCGAACCAATCCCGATCCATTACTGTGCCCCCTGCCGGATTTCGCGATCCTCAAGTGTAACAACAAGACC
 TTCGACGGGAAGGGCCTGTGCACCAACGTGACGACGGTGCAGTGCACCCATGGCATCCGCCCCGTCGTGAG
 CACCCAGCTGCTGCTGAACGGGTCCCTGGCTGAGGAGGAGGTGGTGATCCGGTCGGACAACCTTCATGGACA
 ACACCAAGACAATCATCGTCCAGCTGAACGAGTCTGTGGCGATTAACTGTACCCGGCCTAACAACAACACC
 CGTAAGGGCATCCACATCGGGCCTGGACGGGCTTCTATGCCGCCGCAAGATCATCGGGCAGATCCGGCA
 GGCCCATTTGCAACCTCTCCCGCGCCAGTGGGAATAACACCCTGAAGCAGATCGTGATCAAGCTGAGAGAGC
 ACTTTGGAACAAGACCATCAAGTTCAATCAGAGTTCTGGCGGAGACCCGAGATCGTGCGGCACTCCTTC
 AACTGCGGGGGCGAGTTCTTCTACTGCGATACGACACAGCTCTTCAACTCCACCTGGAACGGCACCGAGGG
 CAACAACACAGAGGGAACTCCACTATCACCTCCCTTGCCGCATCAAGCAGATCATCAACATGTGGCAGG
 AGGTGGGAAAGGCCATGTATGCCCCCCCCATCGGGGGCCAGATCCGCTGCTCCTCAACATCACCGGCCTG
 CTGCTCACCAGAGACGGGGGCACCGAGGGCAACGGCACGGAGAACGAGACGGAGATCTTCAGGCCCCGGCG
 CGGCGACATGAGGGATAACTGGCGGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGTGGAGCCGCTCGGCG
 TGGCCCCCACC CGGGCCAAGCGCCGCTCGTGACAGAAATGGGTGCCCGAGCTTCGGTACTGTCTGGTGGA
 GAGCTGGACAGATGGGAGAAAATTAGGCTGCGCCCGGAGGCCAAAAGAAATACAAGCTCAAGCATATCGT
 GTGGGCCCTCGAGGGAGCTTGAACGGTTTGCCGTGAACCCAGGCCCTGCTGGAAACATCTGAGGGATGTGCGC
 AGATCCTGGGGCAATTGCAGCCATCCCTCCAGACCGGGAGTGAAGAGCTGAGGTCTTGTATAACACAGTG
 GCTACCCTCTACTGCGTACACCAGAGGATCGAGATTAAGGATACCAAGGAGGCCTTGGACAAAATTGAGGA
 GGAGCAAAACAAGAGCAAGAAGAAGGCCACGACGAGCTGCTGACACTGGGCATAGCAACCAGGTATCAC
 AGAATATCCTATTGTCCAAAACATTAGGGCCAGATGGTTTATCAGGCCATCAGCCCCCGGACGCTCAAT
 GCCTGGGTGAAGGTTGTGCAAGAGAAGGCCCTTTCTCCTGAGGTTATCCCATGTTCTCCGCTTTGAGTGA
 GGGGGCCACTCCTCAGGACCTCAATACAATGCTTAATACCGTGGGCGGCCATCAGGCCGCCATGCAAAATGT
 TGAAGGAGACTATCAACGAGGAGGCAGCCGAGTGGGACAGAGTGCATCCCGTCCACGCTGGCCCAATCGCG
 CCGGACAGATGCGGGAGCCTCGCGGCTCTGACATTGCCGGCACCACTCTACACTGCAAGAGCAAAATCGG

ATGGATGACCAACAATCCTCCCATCCCAGTTGGAGAAATCTATAAACGGTGGATCATTCTCGGTCTCAATA
 AAATTGTTAGAATGTACTCTCCGACATCCATCCTTGACATTAGACAGGGACCCAAAGAGCCTTTTAGGGAT
 TACGTCGACCGGTTTTATAAGACCCTGCGAGCAGAGCAGGCCTCTCAGGAGGTCAAAAAGCTGGATGACGGA
 GACACTCCTGGTACAGAACGCTAACCCCGACTGCAAAACAATCTTGAAGGCACTAGGCCCGGCTGCCACCC
 TGGAAAGAGATGATGACCGCCTGTGAGGGAGTAGGCGGACCCGGACACAAAGCCAGAGTGTGATGGTGGGT
 TTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTT
 AAAAGAAAAGGGGGGACTGGAAGGGCTAATCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCT
 ACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTGAGATATCCACTGACC
 TTTGGATGGTGTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGAGAGAACAC
 CAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAGAGAGAAGTGTAGAGTGGAGGTTTG
 ACAGCCGCCTAGCATTTTCATCAGTGGCCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAG
 CCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAGCTGCTTGTACCAATTGCTA
 TTGTAAAAAGTGTGTGCTTTTATTGCCAAGTTTGTGTTTCATAACAGCTGCCTTAGGCATCTCCTATGGCAGGA
 AGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCC
 ACCTCCCAATCCAAAGGGGAGCCGACAGGCCCGAAGGAATAA

Amino acid sequence of antigen:

MAEQLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNWATHACVPTDPNPQEVVLGNVTEYFNMWKNM
 VDQMHEDIISLWDQSLKPCVKLTPLCVTLDCDDVNTNSTTTTTNNGWTGEIRKGEIKNCSFNITTSIRDKV
 QKEYALFYNLDVVPIDDDNATTKNKTTRNFRLIHCNSSVMTQACPKVSFEPIPIHYCAPAGFAILKCNKKT
 FDGKGLCTNVSTVQCTHGIRPVVSTQLLNLGSLAEEVVIRSDNFMNTKTIIIVQLNESVAINCTRPNNNT
 RKGIIHIGPGRFYAARKIIGDIRQAHCNLSRAQWNNTLKKQIVIKLREHFGNKTIKFNQSSGGDPEIVRHSF
 NCGGEFFYCDTTQLFNSTWNGTEGNTEGNSTITLPCRIKQIINMWQEVGKAMYAPPIGGQIRCSSNITGL
 LLTRDGGTEGNGTENETEIFRPGGDMRDNRSELYKYKVVKVEPLGVAPTRAKRRVVQRMGARASVLSGG
 ELDRWEKIRLRPGGKKKYKLKHIVWASRELERFAVNPGLLETSEGCRIILGQLQPSLQTGSEELRSLYNTV
 ATLYCVHQRIEIKDTKEALDKIEEBQNKSKKKAQQAADTGHSNQVSQNYPIVQNIQGMVHQAI SPRTL N
 AWWKVVEEKAFSPFVPMFSALSEGATPQDLNMLNTVGGHQAAMQMLKETINEEAAEWDRVHPVHAGPIA
 PGQMRPGRSDIAGTTSTLQEQIGWMTNNPPIPVGEIYKRWIILGLNKIVRMYSPTSILDIRQGPKEPFRD
 YVDRFYKTLRAEQASQEVKNWMTETLLVQANPDCKTILKALGPAATLEEMMTACQGVGGPGHKARVLMVG
 FPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDWIYHTQGYFPDWQNYTPGPGVRYPLT
 FGWCYKLVPEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFD SRLAFHHVARELHPEYFKNCTSE
 PVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITAAALGISYGRKKRRQRRRPPQGSQTHQVSLSKQP
 TSQSKGEPTGPKE

Figure 13



Sequence of insert:

```

ATGGCCGAGCAGCTGTGGGTCAACGCTCTACTACGGCGTGCCTGTGTGGAAGGAGGCCACGACCACCCTCTT
CTGCGCGAGCGACGCCAAGGCCTACGACACGGAAGTGCATAACGTGTGGGCGACGCATGCTTGCGTGCCTA
CGGACCCCCAACCCCCAGGAGGTGGTGTCTGGGAAACGTGACCGAGTACTTCAACATGTGGAAGAATAACATG
GTGGATCAGATGCACGAGGACATCATCTCTGTGTGGGACCAGTCCCTGAAGCCCTGCGTGAAGCTGACGCC
TCTCTGCGTGACACTGGACTGTGACGACGTCAACACCACCAACAGCACTACCACCACCAGCAACGGCTGGA
CCGGAGAGATTCCGAAGGGCGAGATCAAGAACTGCTCCTTCAATATCACGACCTCGATCAGAGACAAGGTG
CAGAAGGAATACGCGCTGTTTTATAATCTCGATGTGGTCCCCATCGACGACGACAATGCCACCACCAAGAA
CAAGACGACGCGTAATTTAGACTCATTCACCTGCAACAGCAGCGTCATGACGACGGCCTGCCCAAGGTGT
CCTTCGAACCAATCCCGATCCATTACTGTGCCCCCTGCCGGATTCCGCGATCCTCAAGTGAACAACAAGACC
TTCGACGGGAAGGGCCTGTGCACCAACGTCAGCACGGTGCAGTGCACCCATGGCATCCGCCCCGTCGTGAG
CACCCAGCTGCTGCTGAACGGGTCCCTGGCTGAGGAGGAGGTGGTGATCCGGTCGGACAACCTTCATGGACA
ACACCAAGACAATCATCGTCCAGCTGAACGAGTCTGTGGCGATTAACTGTACCCGGCCTAACAACAACACC
CGTAAGGGCATCCACATCGGGCCTGGACGGGCCTTCTATGCCGCCCGCAAGATCATCGGCGACATCCGGCA
GGCCCATTTGCAACCTCTCCCGCGCCCAAGTGAATAACACCCTGAAGCAGATCGTGATCAAGCTGAGAGAGC
ACTTTGGAAACAAGACCATCAAGTTCAATCAGAGTTCTGGCGGAGACCCCGAGATCGTGCGGCACCTCCTTC
AAGTGCGGGGGCGAGTTCTTCTACTGCGATACGACACAGCTCTTCAACTCCACCTGGAACGGCACCGAGGG
CAACAACACAGAGGGAACTCCACTATCACCTCCCTTGCCGCATCAAGCAGATCATCAACATGTGGCAGG
AGGTGGGAAAGGCCATGTATGCCCCCCCCATCGGGGGCCAGATCCGCTGCTCCTCCAACATCACCGGCCTG
CTGCTCACCGAGAGACGGGGCACCGAGGGCAACGGCACGGAGAACGAGACGGAGATCTTCAGGCCCGGCGG
CGGCGACATGAGGGATAACTGGCGGAGCGAGCTGTACAAGTACAAGGTGGTGAAGGTGGAGCCGCTCGGCG
TGGCCCCCACC CGGCCAAGCGCCGCGTGTGACAGAGAATGGGTGCCCGAGCTTCGGTACTGTCTGGTGA
GAGCTGGACAGATGGGAGAAAATTAGGCTGCGCCCGGGAGGCAAAAAGAAATACAAGCTCAAGCATATCGT
GTGGGCCTCGAGGGAGCTTGAACGGTTTGCCGTGAACCCAGGCCTGCTGGAACATCTGAGGGATGTGCGC
AGATCCTGGGGCAATTGCAGCCATCCCTCCAGACCGGGAGTGAAGAGCTGAGGTCTTGTATAACACAGTG
GCTACCCTCTACTGCGTACACCAGAGGATCGAGATTAAGGATACCAAGGAGGCCTTGGACAAAATTGAGGA
GGAGCAAAAACAAGAGCAAGAAGAAGGCCACGAGGCAGCTGCTGACACTGGGCATAGCAACCAGGTATCAC
AGAAGTCTCTATTGTCCAAAACATTCAGGGCCAGATGGTTTCATCAGGCCATCAGCCCCCGGACGCTCAAT
GCCTGGGTGAAGTTGTGCAAGAGAAGGCCCTTTCTCTGAGGTTATCCCCATGTTCTCCGCTTTGAGTGA
GGGGGCCACTCCTCAGGACCTCAATACAATGCTTAATACCGTGGGCGGCCATCAGGCCGCCATGCAATGT
TGAAGGAGACTATCAACGAGGAGGCAGCCGAGTGGGACAGAGTGCATCCCGTCCACGCTGGCCCAATCGCG
CCCGGACAGATGCGGGAGCCTCGCGGCTCTGACATTGCCGGCACCACTCTACACTGCAAGAGCAAATCGG
ATGGATGACCAACAATCCTCCCATCCAGTTGGAGAAATCTATAAACGGTGGATCATTCTCGGTCTCAATA
AAATTGTTAGAATGTACTCTCCGACATCCATCCTTGACATTAGACAGGGACCCAAAGAGCCTTTTAGGGAT

```

TACGTCGACCGGTTTTATAAGACCCTGCGAGCAGAGCAGGCCTCTCAGGAGGTCAAAAACCTGGATGACGGA
 GACACTCCTGGTACAGAACGCTAACCCCGACTGCAAAACAATCTTGAAGGCACTAGGCCCGGCTGCCACCC
 TGGGAAGAGATGATGACCGCCTGTGAGGGAGTAGGGCGGACCCGGACACAAAGCCAGAGTGTTGATGGGTGGC
 AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCAGCAGC
 AGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACAAGTAGCAATACAGCAGCTA
 CCAATGCTGCTTGTGCCTGGCTAGAAGCACAAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTA
 CCTTTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAGAAAAGGGGGGACTGGA
 AGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCC
 CTGATTGGCAGAACTACACACCAGGGCCAGGGGTGAGATATCCACTGACCTTTGGATGGTGCTACAAGCTA
 GTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCTGTGAG
 CCTGCATGGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTTCATC
 ACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCTAGACTAGAG
 CCCTGGAAGCATCCAGGAAGTCAGCCTAAAACCTGCTTGTACCAATTGCTATTGTAAAAAGTGTGCTTTCA
 TTGCCAAGTTTGTTCATAACAGCTGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAA
 GACCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCAAAGGGGAG
 CCGACAGGCCCGAAGGAATAA

Amino acid sequence of antigen:

MAEQLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNWATHACVPTDPNPQEVVLGNVTEYFNMWKNM
 VDQMEDIISLWDQSLKPCVKLTPLCVTLDCDDVNTNSTTTTTNSGWTGEIRKGEIKNCSEFNITTSIRDKV
 QKEYALFYNLDVVPIDDDNATTKNKTTRNFRLIHCNSSVMTQACPKVSFEPPIPIHYCAPAGFAILKCNKKT
 FDGKGLCTNVSTVQCTHGIRPVVSTQLLNGLSLAEDEVIRSDNFMNDNTKTIIVQLNESVAINCTRPNNNT
 RKGIIHIGPGRAFYAARKIIGDIRQAHCNLSRAQWNNTLQIVIKLREHFGNKTIKFNQSSGGDPEIVRHSF
 NCGGEFFYCDTTQLFNSTWNGTEGNNTTEGNSTITLPCRIKQIINMWQEVGKAMYAPPIGGQIRCSSNITGL
 LLTRDGGTEGNGTENETEIFRPGGDMRDNRSELYKYKVVKVEPLGVAPTRAKRRVVQRMGARASVLSGG
 ELDRWEKIRLRPGGKKKYKLKHIVWASRELERFAVNPGLLETSEGCRQILGQLQPSLOTGSEELRSYNTV
 ATLYCVHQRIEIKDTKEALDKIEEEQNKSKKKAQQAADTGHSNQVSQNYPIVQNIQGMVHQAI SPRTL N
 AWWKVVEEKAFSPEVIPMFSALSEGATPQDLNLTMLNTVGGHQAAMQMLKETINEEAAEWDRVHPVHAGPIA
 PGQMREPRGSDIAGTTSTLQEQIGWMTNNPPIPVGEIYKRWIILGLNKIVRMYSPTSILDIRQGPKEPFRD
 YVDRFYKTLRAEQASQEVKNWMTETLLVQANPDCKTILKALGPAATLEEMMTACQGVGGPGHKARVLMGG
 KWSKSSVVGWPTVRRMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQEEEEVGFPVTPQV
 PLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDWYHTQGYFPDWQNYTPGPGVRYPLTFGWCYKL
 VPVEPDKVEEANKGENTSLHLPVSLHGMDDPEREVLEWRFD SRLAFHHVARELHPEYFKNCTSEPVDPRLE
 PWKHPGSQPKTACTNCYCKKCCFHCQVCFITAALGISYGRKKRRRQRRRPPQGSQTHQVSLSKQPTSQSKGE
 PTGPKE

A schematic representation of the constructs and associated expression data is shown below

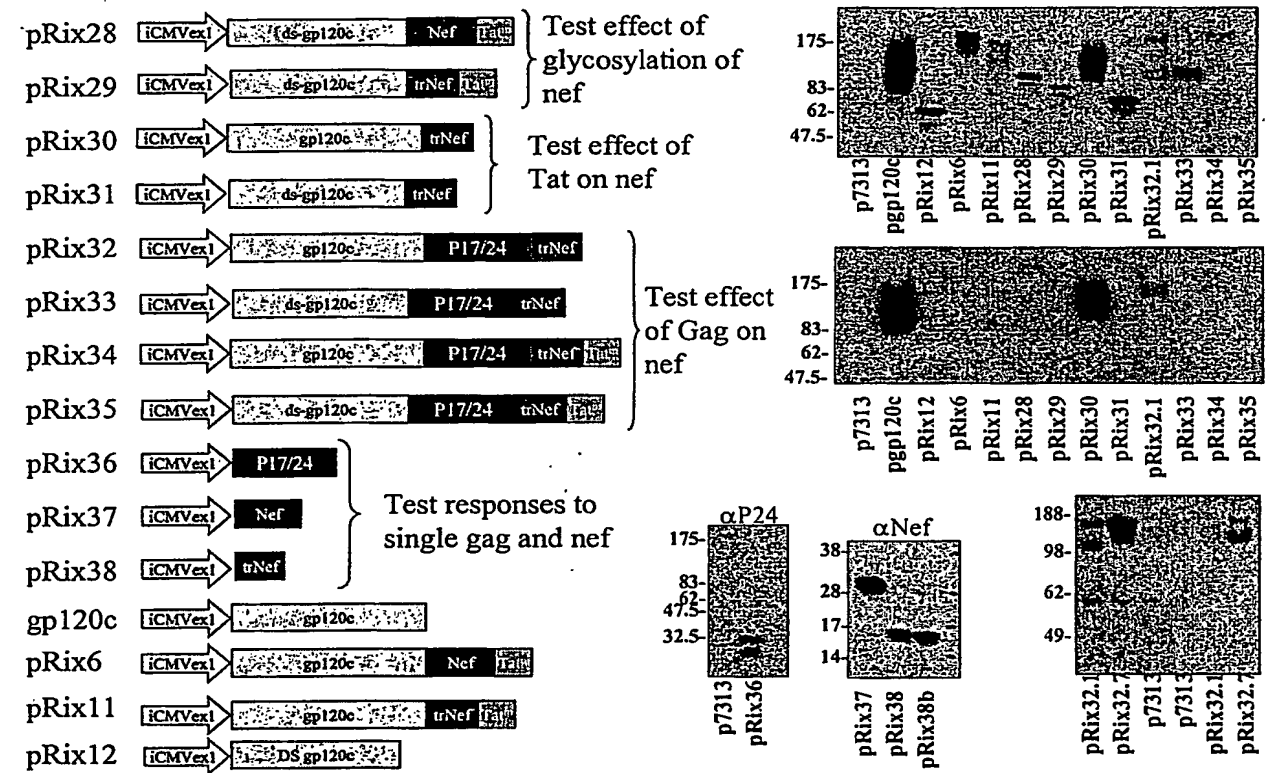


Figure 15

Responses to Gp120 peptide by IFN-gamma ELIspot at 7 days post-boost (Day 35)

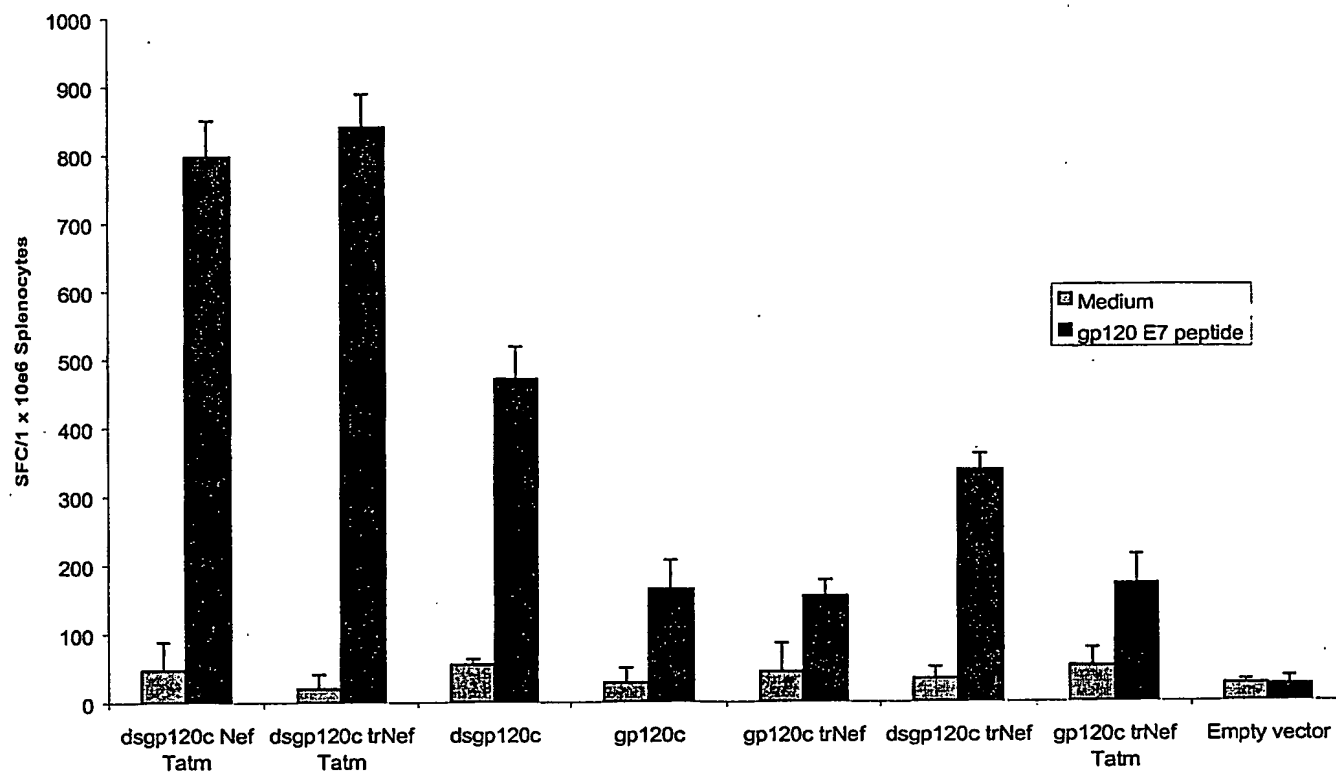
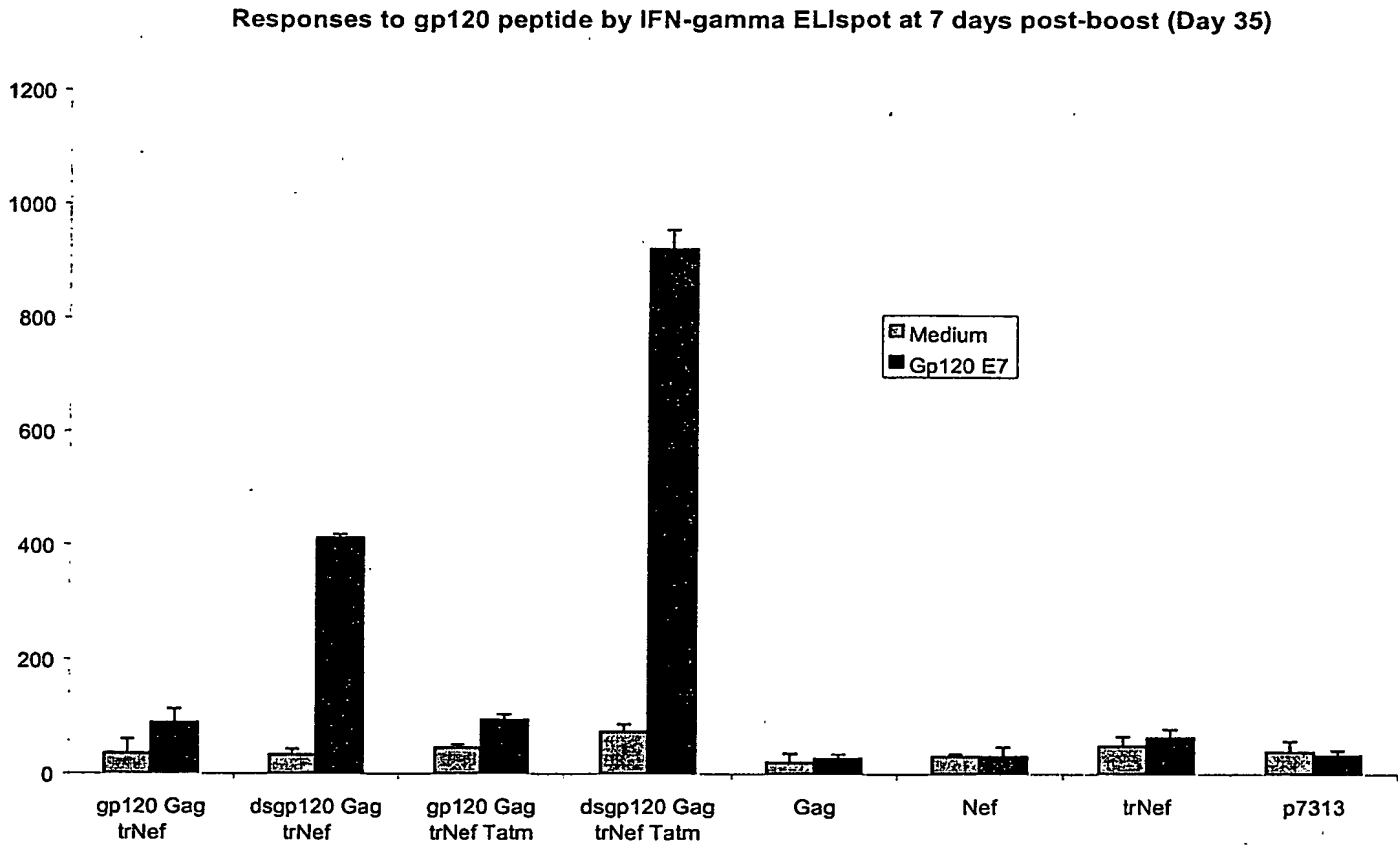


Figure 16



PCT Application

EP0312402



**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☒ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☒ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.